Transcriptomic Analysis of Barley Plant Responses to Cold Stress

Research Project

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Abstract

Previous molecular and genomic studies have shown that several group genes in Arabidopsis with various functions are induced by cold stresses, and that various transcription factors are involved in the regulation of stress-inducible genes which contribute to an increase in cold tolerance. Here, we present the results of transcriptome analysis indicating the existence of genes of potential importance to cold stress and multiple low-temperature regulatory pathways in addition to the cold response pathway in barley.

To identify cold-responsive genes, global expression profiling was performed on barley plants subjected to stress treatments of 4°C in root. RNA samples were collected separately from leaves and roots after 4 weeks cold stress treatment. The expression profiling was conducted with barley Genome Affymetrix microarray with probe sets for approximately 53,030 "unigenes". A total of 2577 genes with greater than 2-fold change over control were identified as being cold responsive, with transcripts for 185 genes increasing ten-fold or more at one or more time points during 4 weeks experiment. These results suggest that extensive up/down-regulation of gene expression occurs during cold acclimation, and about 7% of the transcriptome is sensitive to regulation by common stress conditions. AGL19 was found to be the most important regulator in the vernaliztion response pathway. FAD7 was found to be very active in many pathways, indicating the gene can protect plant cell from cold damage. FAD7 is under the control of Salicylate and the existing of Propyl Gallate which acted as antioxidant suppressed the expression of FAD7. Other genes, ICE1, LTI30 and RAB18 showed a highly expressed in root cooling treatment and have significant strong links with other cold responsive genes which can enhance the cold and freezing tolerance in Barley. SEX1 was identified to be the main regulator in the starch catabolic process, and was highly expressed in root cooling treatment. This observation suggests that fructan metabolism may also occur during root cooling, offering further potential for the control of sucrose gradients in the leaf.

ABBREVIATIONS

ABA – Abscisic Acid

AGL19 –AGMOUS0LIKE 19

CBFs–C-repeat (CRT)-binding factors

CBF3–ERF/AP2 transcription factor family

COR47–COLD REGULATED 47

DD–Differential Display

EST–Expressed Sequence Tags

FAD7–FDA7-FATTY ACID DESATURASE 7

HOS1–HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1

ICE1– INDUCER OF CBF EXPRESSION1

LTI30–LOW TEMPERATURE-INDUCED 30

RAB18–RESPONSIVE TO ABA 18

SAGE–Serial Analysis of Gene Expression

SEX1–STARCH EXCESS 1

TF – Transcription Factor

Contents

1 INTRODUCTION

1.1 Background

Food Security is of central important in UK and is considered as a strategic preferential area. To enhance food security, it's of high necessary to understand and moreover, optimize the response of crop plants to make them can cope with climate change. Barley is the most widely grown cereal grain in the world for more than 35% of the worlds' population. Therefore Barley is believed to be one of the most important arable crops in the UK and EU (FAO, 2007).

Developing crops which can tolerate environmental stresses, and can also maintain productivity, is a critical requirement for sustainable agriculture in this era (M. Jenks *et al*., 2005). Many challenges are ahead, from burgeoning population to diminishing arable land, we need to feed more people on limited agricultural land. Therefore, finding ways to nourish both people and the planet with environmentally sustainable methods and to cope with climate change is very vital (K. Shameer *et al.*, 2009).

Recent advances in characterizing the barley genome give the promise of enabling rapid, reliable determination of alleles that allow more accurate targeting of genes (Seo, M *et al*., 1997). The identification of the genes involved in the cold stress response will be crucial for breeding crops able to cope with the effects of climate change (Zheng, L *et al*., 1993). It is a timely moment to develop a predictive model to investigate the molecular mechanisms of barley cold acclimation and cold stress.

1.2 Transcriptome and Gene Regulatory Network

Transcriptome analysis of large numbers of genes will allow us to specify the identity

and level of expression of target genes that are associated with cold stress response for a knowledge-based plant breeding (Roosens, N.H. *et al*., 1999). However, the absolute level of expression of these genes cannot be determined directly for the co-ordinated regulation. Gene interaction studies can give critical insight into the regulation of the cold response.

A gene regulatory network presents regulations among the genes. Generally, regulatory networks are created in two common cases: when a regulator produces its production, and this production requires another regulator, these two regulators and the production may form a regulatory network; when one regulator's activity is regulated by another regulator, directly, these two regulators may form a regulatory network. From this perspective, the whole regulator network which exists in the genetic network is quite complicated.

To establish a topology of the network from bench experiments is quite complex and usually may take a lot of time. In this case, Gene-expression data is an ideal datasets from which networks can be inferred (Hiroyuki Toh and Katsuhisa Horimoto, 2002). As we can know, even if very excellent predictive power is exist, the power of getting an accurate inference of some techniques is quite low. Therefore, we need more complex models that have the capacity of establishing both high prediction and inferential power. (Xiuwei Zhang *et al*., 2009)

Genetic algorithm is one of the computational models. Compared with other methods, it has not been used for a very long time, but still, it has been used widely, and reports show that it's quite effectively (John Dougherty *et al*., 2008). It has been used as search methods and also, has been used to build up models for evolutionary systems (Matthew J. Beal and Praveen Krishnamurthy). As computational models, in applying genetic algorithms, computer can store the information of binary strings (J. Pearl, 1988). In the long term, strings are been selected in an evolutionary way which

been carried out by the computational setting according to what we expect then finally, we can get complex and interesting structures (Adriano V. Werhli *et al*., 2006). Once we get these structures, they may lead us a way to give solutions to problems, strategies for visualizing images. (Stephanie Forrest, 1996)

The general purpose of inferring gene regulatory network is to extract the expression features, activation and inhibitions from the changes of gene expressions from trancriptome data (N. Friedman *et al*., 2000). Recently, researches focus on the reverse engineering methods and try to understand the complex interactions that are directly affected by the gene networks. Several mathematical methods for modelling the gene regulatory networks have been proposed such as Boolean networks (Tatsuya Akutsu *et al*., 2000), Bayesian networks (Friedman N *et al*. 2000), Artificial Neural Networks (ANNs) (Bar-Yam and Yaneer 2003), and Time-Series Network Inference (TSNI). Bayesian networks is a method for computational modelling of GRNs from expression data since they are able to represent the network both graphically and quantitatively and thus are relatively easy to interpret by non-statisticians (Daxin Jiang *et al*., 2004). A common problem in such kind of Baysesian and TSNI learning approaches is that only a small number of genes can be modelled by such data-driven methods (S. Y. Rhee *et al*., 2006).

With genomic sequence data available, bioinformatics tools have been valuable for analysing large scale of genes and understanding gene regulation (K. Shameer *et al.*, 2009). For instance, transcriptome analysis using Microarray technology has identified several genes that are induced by cold stress (Kazuo Shinozaki *et al*., 2003).

Primary carbon metabolism in the leaves of higher plants is tightly regulated for two contrasting reasons. “Feed-forward” regulation is directed towards optimising short-term carbon fixation under fluctuating external conditions (particularly irradiance). This regulation operates (mainly at the fine control level) to balance the

rates of RuBP regeneration, triose phosphate export and starch synthesis in order to optimise primary carboxylation in terms of the supply of CO_2, ATP and reductant (for a general discussion, see Stitt, 1996). By contrast, “Feed-back” regulation ensures that there is medium and long-term linkage between the demand for carbon in sink tissues and its supply by source leaves (John F. Farrar *et al*., 1998). Photosynthate that is produced in excess of current demand can be repartitioned into storage carbohydrates such as starch and fructans (John T. Farrara *et al.*, 2000) and photosynthetic capacity can be reduced by down-regulation of key genes associated with assimilate accumulation (Jang and Sheen, 1994).

The main goal of this project is to identify target genes involved in cold stress response and carbon metabolism in barley. We will use genomics information and transcriptomic data as the primary input to reconstruct the gene regulatory network and then to test and refine by gene approaches.

1.3 General Stress with Signal Transduction Pathway

Signal ignition can trigger the generic signal transduction pathway. Second messengers would be triggered subsequently (Strizhov, N *et al*., 1997). The content of intracellular Ca^{2+} can be modulated by second messengers which resulting to start a protein phosphorylation cascade. This could eventually targets proteins directly involved in cellular protection or transcription factors controlling specific sets of stress regulated genes (Liming Xiong *et al*., 2002).

Many molecules have been identified as regulatory molecules. For instance, Plant hormones abscisic acid (ABA), ethylene, salicylic acid (SA) are all belong to regulatory molecules (Stockinger, E.J *et al*., 1999). These regulatory molecules are under control of the products generated by stress regulated genes. Therefore, these regulatory molecules can start a second round of signaling. Though often with different components to participate, this second round signaling is following the previous

generic pathway (Liming Xiong *et al*., 2001).

Some molecules do not transfer the signal directly, they are more likely to work as assembly components or participant in delivery and modification. These molecules are also very important in the accurate transmission of stress signals (Xiong and Zhu, 2001). They are believed to provide the adequate spatial and temporal coordination for all signaling molecules during signal transduction (Liming Xiong *et al*., 2002).

The Expression Sets of Stress Regulated Genes are different in Roots and Leaves

Basically, different sets of specialized cells exist in roots and leaves. Research has been done to find out how different the stress response process would be between different tissues (Savoure, A *et al*., 1997). Report has shown that roots and leaves have very different transcriptome response to cold, salt, and osmotic stresses (Joel A *et al*., 2002). In the study of Joel A, they have found evidence to support this opinion that 86% of the cold induced changes genes are not shared between root and leaves (Joel A *et al*., 2002). This may suggest that in different tissues, the response to cold stress would be very much different and with the unique response pathway we may be able to better understand the whole stress response process in plants (Liu, Q *et al*., 1998).

ABA has been identified to be the most important regulatory molecule during the signal transduction pathway. And all the genes involved in stress response can be generally divided as ABA-dependent and ABA in-dependent.

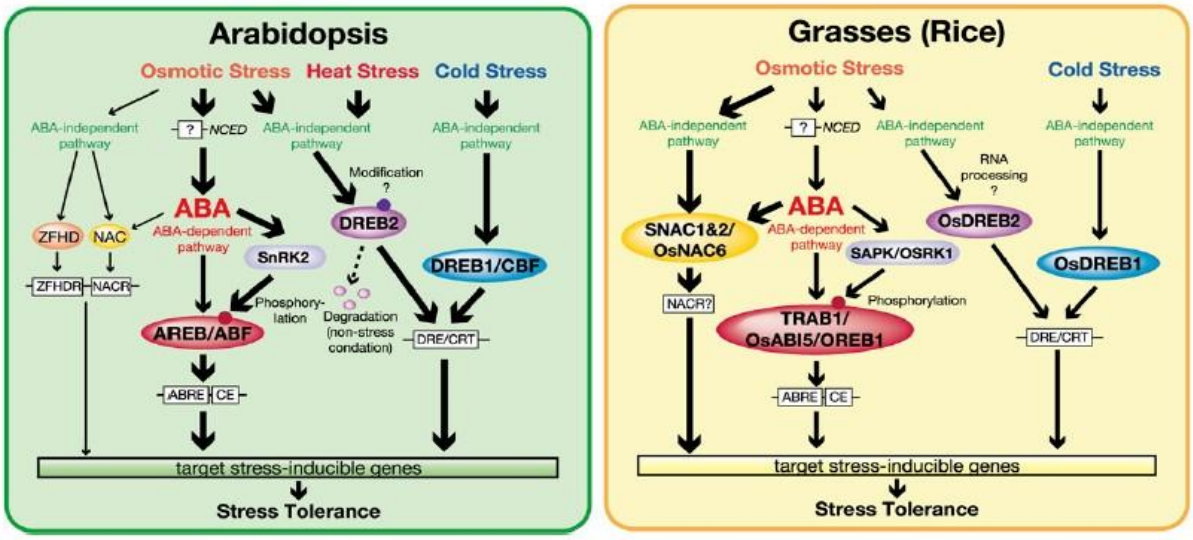


Figure 1. ABA-independent and ABA-dependent signal transduction pathways response to abiotic stresses in Arabidopsis and Grasses (Kazuo Nakashima *et al*., 2009).

1.4 Cold-Stress Response Related Genes

As an adverse environment, cold stress can cause effects on plant growth and seed production, meanwhile, plants can response and adapt to cold stress through various biochemical and physiological processes (Kazuo Shinozaki *et al*., 2003).

The expression of a single gene is determined by a number of different transcription factors, and the ratio of their concentrations may be vital, particularly if competing positive and negative factors are involved (D.S LATCHMAN, 1993). As many transcription factor (TF) genes were found among the stress-inducible genes, we can know that there are various transcriptional regulatory mechanisms in the cold stress signal transduction pathways (Kazuo Shinozaki *et al*., 2003)

TFs are master regulators that control gene clusters. A single TF can control the expression of many target genes through specific binding of the TF to the cis-acting

element in the prompters of respective target genes (Kazuo Nakashima *et al*., 2009). From this point of view, focusing on the transcriptome analysis including transcription factor, will provide better understanding of how these respond processes occur (K. Shameer *et al.*, 2009).

Molecular and genomic analyses have shown that several different transcriptional regulatory systems are involved in stress-responsive gene induction (Kazuo Shinozaki *et al*., 2003). The *INDUCER OF CBF EXPRESSION1* (*ICE1*) genes was identified through the map based cloning of the *Arabidopsis ice1* mutation (Gilomour SJ *et al*., 1998). Over expression *ICE1* in transgenics resulted in improving freezing tolerance, supporting an important role for *ICE1* in the cold-stress response (Kazuo Shinozaki *et al*., 2003). Many cold regulated genes in Barley have been found (Cristina Crosatti *et al*., 2006).

In addition, many genes involved in chromatin level and posttranscriptional regulation were also cold regulated. A number of genes have been identified for the biosynthesis or signaling of plant hormones, such as abscisic acid, gibberellic acids, and auxin, are regulated by cold stress, which are of potential importance in coordinating cold tolerance with growth and development (Mie Kasuga *et al*., 1999).

1.4.1 DREB1/CBF and DREB2 Regulons in Cold Stress Response

DRE is known as Dehydration-Responsive Element. It contains the core sequence A/GCCGAC, which has been identified as an ABA-independent cis-acting element (Ishitani, M *et al*., 1997). It has been proven to be very important to regulate the expression of gene in cold stress response biological processes (Yamaguchi-Shinozaki and Shinozaki, 1994). DRE core motif have been investigated to exist in a similar cis-acting elements which is named C-repeat (CRT) and low temperature responsive element. CRT and LT responsive element are both present in genes that can be

induced by cold or even freezing stress (Baker *et al*., 1994; Jiang *et al*., 1996).

Most DREB1 genes have shown that they are very sensitive to temperature falling and can response to cold stress very quickly (Thomashow, M.F, 1994). Overexpression of DREB1/CBF homologous can enhance the cold and freezing tolerance of plants (Clauda Jonak *et al*., 1996). Meanwhile, the overexpressor lines of these genes can improve the sensitivity to cold stress of plants (Kazuo Nakashima *et al.*, 2009).

DREB1/CBF genes have not only been found in Arabidopsis but also in various kinds of plants especially grasses. Research results have shown that the DREB1/CBF gene can work as transcription factors which function are similar to the Arabidopsis DREB1A transcription factor (Kazuo Nakashima *et al.*, 2009).

1.4.2 codA gene and Accumulation of betaine to Enhance the Cold Tolerance of Plant

codA has been reported that can enhance the plants tolerant to both salt and cold stress (Hidenori Hayashi *et al*., 1997). To realize the tolerance enhancement, codA should work together with betaine. As the expression of codA is under control of betaine, the concentration of betaine is very important to induce the expression of codA (Rathinasabapathi, B *et al*., 1994). The accumulation of betaine could be considered as the very first step of this signal pathway (Weretilnyk, E.A. and Hanson, A.D, 1990).

In barley, betaine has been detected to be synthesized in the stroma of chloroplasts. This may reveal that chloroplasts is the major place that betaine would accumulate in (Brouquisse *et al*., 1989; Weigel *et al*., 1986). This may suggest that codA is much more active in leaves than that in roots (Deshnium, P *et al*., 1995).

When the accumulation of betaine in plant cells reach a certain level (the threshold would be vary in different plants), the tolerance to low temperature would be enhanced of significant level (Stockinger, E.J *et al*., 1997). It's most likely happen with the reason that the protection of photosynthesis in plants against low temperature photo-inhibition of recovery from the photo-inhibited state by the accumulation of betaine in chloroplast (Hidenori Hayashi *et al*., 1997).

1.4.3 HOS1 and the Negative Regulation of Many Cold Inducible Genes in Plants

Some cold responsive genes, such as RD29A, COR47, COR15A, KIN1, have been investigated as overexpressed when they are regulated by HOS1 (Jonak, G *et al*., 1996). Though these genes would be induced by many other abiotic stresses and accumulation of molecules, their expression are enhanced by HOS1 only under cold stress (Stockinger, E.J *et al*., 1997). This suggests that cold stress is the major trigger to connect HOS1 with these cold responsive genes which resulting to enhance the cold tolerance of plant (Manabu Ishitani *et al*., 1998).

In the study of Manabu Ishitani (1998), evidence was found to indicate that HOS1 locus is a very important regulator of cold signal transduction in plant cells, and it always acted as negative regulator. And also, it has been found has correlation with vernalization response that it may accelerate the development from vegetative growth to reproductive growth when plants are treated with bearable low temperature for certain long period (Kurkela, S and Borg-Franck, M, 1992).

As taken together, HOS1 can negative regulate many cold stress response genes in plants, therefore it has been considered to play critical roles in controlling gene expression under cold stress, freezing tolerance, and flowering time (Manabu Ishitani *et al*., 1998).

1.5 Gene Expression Analysis Platforms

There are many gene expression platforms that can be applied to study gene expression. Most of these platforms are also compatible with microgenomic studies with no or very few modifications. The most widely used gene expression study platforms are Serial Analysis of Gene Expression (SAGE), Expressed Sequence Tags (EST), Differential Display (DD), Microarray and Whole Transcriptome Sequencing (Jayakumar, 2009).

The hybridized Microarray can be excited by a laser and scanned at wavelengths suitable for the detection of the red and green dyes, in this way, we can estimate relative expression levels of genes in both samples from fluorescence intensities and colors of Microarray spots (Helen C. Causton *et al*., 2003).

Then by using a Microarray approach we can obtain a broad overview of the behavior of the barley transcriptome to cold-stress condition (Mark O Winfield *et al*., 2009). The Microarray data got from Affymetrix Genechip can be extracted from scanned GeneChip images and analyzed using Microarray Suite. GeneSpring is the most widely used software for analysising transcriptome data (Mark O Winfield *et al*., 2009).

As a popular and basic data analysis software, GeneSpring has been used widely and frequently by many researchers. With concise working windows and easily operating functions, GeneSpring could lead even the beginners through the process of data analysis without getting lost. The powerful database provided by GeneSpring can sort out what the researcher expect in different ways. Researcher can easily identify the desired targets both through statistically and biologically methods. Users can update the newest updating data to catch up with the most novel research on gene level. As a member of Agilent's GeneSpring Analysis Platform, GeneSpring GX is "an expanding

suite of integrated software applications for system-level research" (*AGILENT,* 2009).

1.6 Hydroponic Cultivation

The Hydroponic Cultivation has been chosen for this experiment to realize the constant and accurate growing temperature circumstance for Barley plants. As a long-developed and world-wide known technique, hydroponic has been used to cultivate plants of many varieties. Without using of soil, the surrounding around the plant root can be controlled as exactly as researchers want. From nutrient elements to temperature, and also illumination, researchers can design their experiments as detailed as possible and carried out them in the most desirable way (J.Benton Jones). Another advantage of hydroponic cultivation is after plant growing period, the collection would be much more easily compared with soil cultivation. In this experiment, we used Long Ashton Solution as the Hydroponic Cultivation Solution. This solution could provide all the Barley plants with the appropriate nutrient elements both in amount and varieties that the plants desire.

1.7 Purpose of the Study:

1. Growing plants: Long Ashton Solution (LAS) will be used to cultivate Barley plants which will be divided into two groups. One group plants will grow at normal condition (both root and leave are exposed at 20 degrees centigrade). Another group plants, the roots will grow in the cold Long Ashton Solution (4 degrees centigrade) and at the same time the leaves will be exposed in normal temperature (20 degrees centigrade).
2. Identifying cold response target genes and regulators using barley Affymetrix genome array.

   The transcriptome data of Barley which will be obtained from Microarray will be analyzed by GeneSpring GX 11. 0. 2. Certain genes (act as regulators or regulate targets) that most relevant to cold stress response will be found. Their functions and interactions will be discussed. With the discussion, it's likely to provide a insight view of how these regulator realize their functions when Barley are treated with low temperature. Moreover, it's hopefully to find out some other genes that related to these most important regulators and how they work in the cold stress response process.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Barley Plants

Barley seeds *(Hordeum vulgare L. cv Klaxon*) were obtained from The John Innes Centre (JIC).

The Barley seeds were sowed in 2 separate Petri dishes with sterile clean filter paper beds. All the seeds were placed properly to allow enough space for each seed in order to avoid oxygen deficit in the process of burgeon. Adding distilled water into Petri dishes and make sure that all the seeds were submerged in distilled water.

15 healthy seedlings were selected to grow in half-strength Long Ashton Solution after 14 days with different temperature treatments (Fig. 2)

Figure 2. 14 days old Barley seedlings were selected for growing in the half-strength Long Ashton Solution

Two treatments were arranged for the study:

1. Control – growing whole plants (from root to leaf) in the LAS at 25 ^{o}C ;
2. Cooling – roots were grown in LAS at 4^{o}C; rest of plants (stem and leaf) were exposed at 25 ^{o}C

At this point, seedlings were transferred to the growth cabinet with a 14 h photoperiod (280) and 22°C day/18°C night temperature (20°C in average) in

South Laboratory (Sutton Bonington Campus, The University of Nottingham). The cooling machine (Fig.3) was maintained in the growth cabinet for the cooling treatment.

2.1.2 Set the Cooling and Normal Growing Conditions

The cooling machine was set up and 9 Barley plants were fixed into the machine.

2.1.2.1 The Cooling Machine

The Cooling Machine was placed in the growth room of South Laboratory (Sutton Bonington Campus, The University of Nottingham).

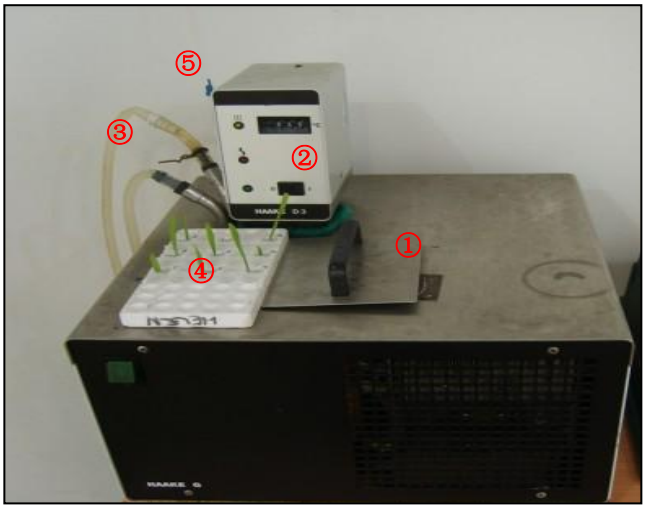


Figure 3. The cooling machine and the designs for providing low temperature growing condition for Barley plants

① The Long Ashton Solution was adding into the machine through here.

② The central controller of this temperature adjustable machine. The temperature was set at 4 degrees centigrade.

③ This tube was designed to realize the circulation of the hydroponic cultivation solution.

④ The Barley Plants were placed on the cube of this machine. With the fulfill solution inside the cube, the roots of these plants can merge into the solution to get the nutrient needed during the growth process.

⑤ The thermometer was inserted into the cube from here to investigate the temperature.

2.1.2.2 Fix the Barley Plants on the Machine

The sterile tubes have been used to fix the plants in. Cut down the covers and buttons of all the tubes, placed every individual plant into one single tube. This process should be extremely careful to protect all the plants from physical wounds which might lead to certain infections by plant pathogen. With the button no longer existed, the root could went through the tube and came into contact with the solution directly and sufficiently. In the same time, the leaves of the Barley plants still been exposed into the room air and with the temperature of average 20 degrees centigrade.

The plastic foam board had been chosen to fix all the plants on. This is due because that the plastic foam is water-proof and also has desirable thermal insulation, which is really important to keep the temperature of the inside solution always stay at 4 degrees centigrade. Placed all the tubes with Barley plants fixed in them into the small holes on the plastic foam board, and make sure there were no gap between the tubes and plastic foam board.

Figure 4. Barley plants were placed into sterile tubes which had the buttons cut down and fixed into a plastic foam board, the roots were allowed to stretch into the Long Ashton Solution freely.

There were 9 Barley Plants in total that grew with their roots merged into the 4

degree centigrade Long Ashton Solution.

2.1.2.3 Normal Growing Condition

5 Barley plants have been placed into tubes which had their covers and buttons cut down. The plastic foam board was used to fix all the tubes on. Two trays were prepared for containing Long Ashton Solution. As a result, the roots could merge into the solution all the time. The solution in the small tray was exposed into air, the temperature of the solution was the same as the room temperature, so the roots and leaves shared the same growing temperature.

2.1.3 Hydroponic Cultivation Solution

The Hydroponic Cultivation Solution used for this experiment was Long Ashton Solution. The elements involved and their required concentrations were different from traditional Long Ashton Solution.

Table 1. Long Ashton Solution that used to provide nutrients for Barley plants

No.	Formula	Amount in 500 (g)	Amount in 1L (g)
①	KNO_3(Potassium Nitrate)	50.5	101.1
②	$Ca(NO_3)_2.4H_2O$ (Calcium Nitrate)	236	472
③	$NaH_2PO_4.2H_2O$ (Sodium Phosphate Monobasic)	52	104
④	$MgSO_4.7H_2O$ (Magnesium Sulfate Heptahydrate)	92	184
⑤	EDTA Fe-Na Salt	18.65	37.30
⑥	**Micronutrients**		
1.	$MnSO_4.H_2O$ (Manganese Sulfate)	1.125	2.25
2.	$CuSO_4.5H_2O$ (Cupric Sulfate)	0.125	0.25
3.	$ZnSO_4.7H_2O$ (Zinc Sulfate)	0.145	0.29

4.	H_3BO_3 (Boric Acid)	1.55	3.1
5.	$NaM_OO_4.2H_2O$ (Sodium Molybdate Dihydrate)	0.06	0.12
6.	NaCl (Sodium Chloride)	2.93	5.86

***** For 1 Liter Long Ashton Solution, there should be 4ml of ① plus 2ml of ②③④ plus 1ml of ⑤⑥ and together with 10mg of Sodium Metasilicate for full strength.

2.1.4 The Collection of Barley Plants

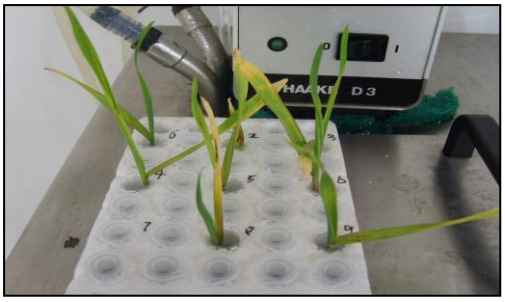


Figure 5. Cooling controlled Barley plants on the day for collecting

Figure 6. Normal controlled Barley plants on the day for collecting

*The cooling controlled group was much weaker than the normal group. Cut every plant with sterile scissors and separated the plant as roots, leaves, and stem. Put each sort of tissue into the corresponding prepared marked tube, placed the tube into liquid nitrogen to prevent the RNA from degradation. All the tubes with tissues in them were stored in freezer with the temperature maintaining at minus 80 degrees. All the tubes that were used to load the plant tissues and scissors, tweezers used to separate the plants should all be sterile before collection.

2.2 Methods

2.2.1 RNA Extraction

TRIzol® Reagent was used to extract total RNA from Barley plants. 8 samples were prepared:

T able 2. RNA samples prepared for Microarray analysis

Sample Code	Tissue	Treated Temperature	Replicate
1	Root	4 ℃	1
2	Root	4 ℃	2
3	Root	20 ℃	1
4	Root	20 ℃	2
5	Leaf	20 ℃ (with root as 4 ℃)	1
6	Leaf	20 ℃ (with root as 4 ℃)	2
7	Leaf	20 ℃	1
8	Leaf	20 ℃	2

2.2.2 RNA Quality Check

2.2.2.1 Agilent Bioanalyzer

Before carrying out Microarray, the RNA samples must be run through the Agilent Gel to check the quality of RNA to make sure that the Microarray can be carried out successfully. All the prepared RNA samples were sent to NASC (The Nottingham Arabidopsis Stoke Centre) to run Agilent Gel.

Table 3. Information about instrument and assay of Agilent Gel RNA Quality Check Task for all the samples

Instrument Name	DE04700474
Firmware	C.01.055
Serial#:	DEO4700474
Type	G2938A
Assay Class	Eukaryote Total RNA Nano
Version	2.4

2.2.2.2 RNA Quality Check Results：

Table 4. Overall RNA Quality Check Results of 8 Samples

Overall Results for 8 Samples		
Samole Code	**RNA Concentration**	**rRNA Ratio [28s/18s]**
1	385 ng/μ l	2.3
2	827 ng/μ l	1.4
3	600 ng/μ l	1.5
4	554 ng/μ l	1.6
5	2.488 ng/μ l	1.0
6	2.930 ng/μ l	0.9
7	827 ng/μ l	1.4
8	2,085 ng/μ l	1.6

For these 8 samples, the RNA concentrations were higher than the request from NASC and also, the rRNA ratios were high enough to do the subsequently steps. For

RNA quality check, all the samples had passed the RNA quality standard for Microarray experiment.

2.2.3 Microarray

In this experiment, we chose The GeneChip ®Barley Genome Array for all the Barley samples. Affymetrix barley chips represents a considerable comprehensive and informative content for barley gene expression research. This GeneChip contains more than 22,500 probe sets representing transcripts from the *Hordeum vulgare* genome (*AFFYMETRIX*, 2003).

The GeneChip Barley Genome Array was designed and funded by the USDA-IFAFS Triticeae Improvement group (R. Wise, T. Close, G. Muehlbauer, R. Wing, and A. Kleinhofs) in collaboration with Affymetrix and the international barley community. A community effort resulted in a significant improvement to sequence quality through better clustering and derived annotations. Sequences used for the Barley Genome Array design were collected from consortia labs submitting EST sequences and by collecting sequences from the NCBI/GenBank non-redundant database. Approximately 400,000 raw barley ESTs were submitted from 84 libraries, and about 350,000 survived quality pruning (*AFFYMETRIX*, 2009).

Stringent CAP3 clustering (-p95 -d60 -f100 -h50) was performed and resulted in 53,030 "unigenes" (26,634 contigs and 26,396 singletons). 25,500 contigs and singletons had complete 3' ends suitable for array design (see HarvEST Triticeae v0.95 and higher). This included all 1,145 known barley genes (including alleles) from the NCBI non-redundant database. The nonredundant cloned gene set was integrated with the EST clusters to aid in scaffolding the ESTs and also to retrieve any rare interesting genes (e.g., Mla, Rar1, Sgt1, Rpg1) for inclusion on the GeneChip microarray. After pruning against an enhanced Triticeae repeat element database (TREP), the exemplar set of 25,500 contigs and singletons was submitted to

Affymetrix for initial computation(*AFFYMETRIX*, 2009).

This GeneChip for barley array can be applied to many aspects:

Malting properties	Pest Control	Disease Control
Nutritional characteristics	Reproductive development	Abiotic Stress Tolerance

To do a Microarray and the corresponding data analysis, here is a general workflow which described in (Helen C. Causton *et al*., 2003):
Array fabrication → preparation of the biological samples to be studied → extraction and labeling of the RNA from the samples → image quantitation (locate the spots in the image and measure their fluorescence intensities) → data normalization and integration (construct the gene expression matrix that describes gene expression values from sets of spot quantitations from different hybridizations) → gene expression data analysis and mining → generation from these analyses of new hypotheses about the underlying biological process (Helen C. Causton *et al*., 2003).

With doing all these steps, we expect to search for clusters of co-expressed genes and to deduce regulatory information and also to advance in the understanding of various functions are induced by cold stress (Chungui Lu, 2009).

The samples were sent to NASC for Microarray analysis and this part of work has been done by Zoe Emerrson. We got the final .CEL files for all the samples to do the GeneSpring data analysis.

2.2.4 GeneSpring Data Analysis

The datas which obtained from Microarray of the 8 samples were imported into GeneSpring GX 11. 0. 2 for comprehensive analysis with the hope to find cold-stress related genes and associated pathways.

2.2.4.1 Settings of Experiment

In this experiment, the following options were selected to set up the GeneSpring GX experiment:

Table 5 . selections of GeneSpring GX analysising modles for setting up data analysis

Modle	**Option**
Experiment type	Affymetrix Expression
Workflow type	Advanced Analysis
Summarization Algorithm	RMA
Baseline	to median of all samples

2.2.4.2 Import datas and Add parameters

The data which got from Microarray of all the 8 samples were imported into GeneSpring GX for future analysising.

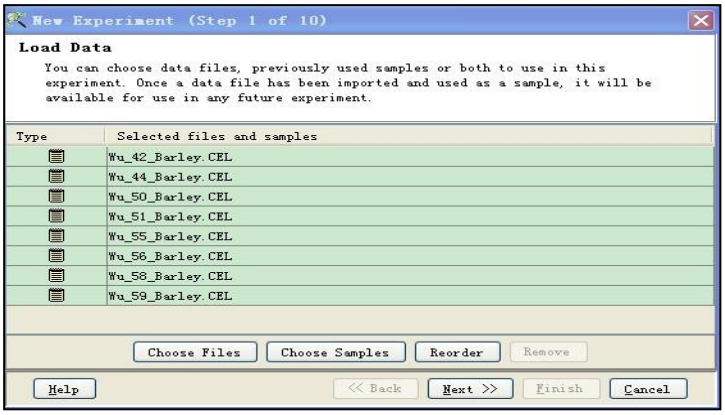


Figure 7. The datas imported into GeneSpring GX for data analysing.

Adding collected tissue and treated temperature and the number of replicates as three parameters for all 8 samples:

Table 6. The parameters added for each sample

Samples	tissue	temperature	replicate
Wu_42_Barley.CEL	root	20	1
Wu_44_Barley.CEL	root	20	2
Wu_50_Barley.CEL	leaf	20(with root as 4)	1
Wu_51_Barley.CEL	leaf	20(with root as 4)	2
Wu_55_Barley.CEL	leaf	20	1
Wu_56_Barley.CEL	leaf	20	2
Wu_58_Barley.CEL	root	4	1
Wu_59_Barley.CEL	root	4	2

Chose the average over replicates in conditions for all the samples, and created a new interpretation, then we got the interpretation plot for all 8 samples. In the same way, the interpretation plot for samples with non-average over replicates in conditions is shown as below:

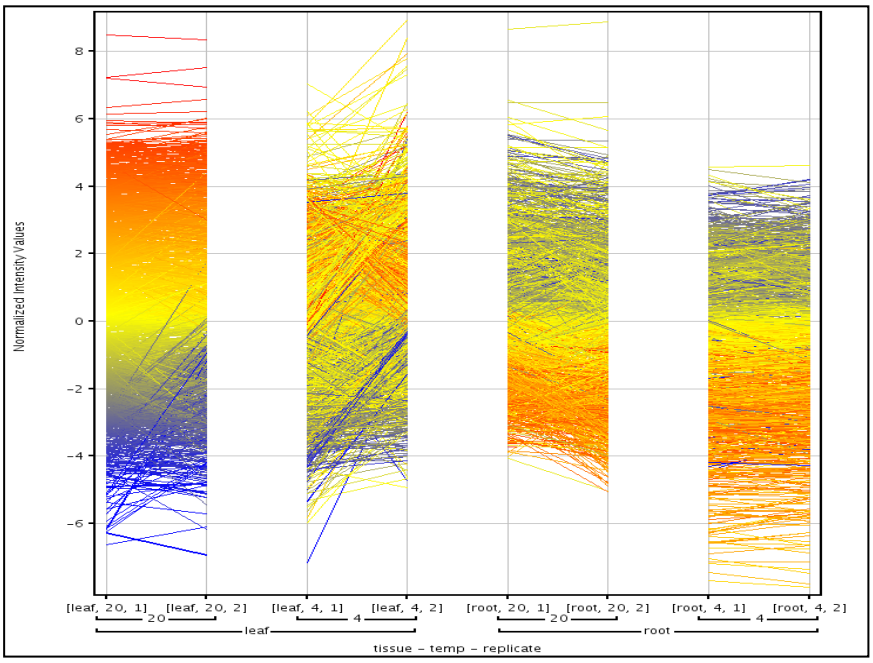


Figure 8. The interpretation plot for all 8 samples with the setting as non-average over replicates in conditions

From this interpretation plot, the comparasions that could be made between the same group (samples collected from the same tissue and treated with the same temperature) replicates can give the clear view that the two replicates have the great similarity on Gene Expression Level.

2.2.4.3 Create New Gene-Level Experiment

With this interpretation for all the 8 samples, the creation of Gene-Level experiment can be made for the 8 samples and the worklflow type should aslo be selected as Advanced Analysis. For another options to create the experiment are listed as below:

Table 7. Selections of GeneSpring GX 11. 0. 2 analysising modles for setting up Gene-Level experiment

Setting	**Option**
Threshold raw signals to	1.0
Normalization algorithm	Percentile Shift
Shift to percentile	75
Baseline	to median of all samples

2.2.4.4 Quality Control on Samples

Principal Componenets Analysis (PCA) could be carried out, with the Correlation Coefficients and Hybridization Controls, it's possible to make sure that the certain two replicates have great similarity on statistical level.

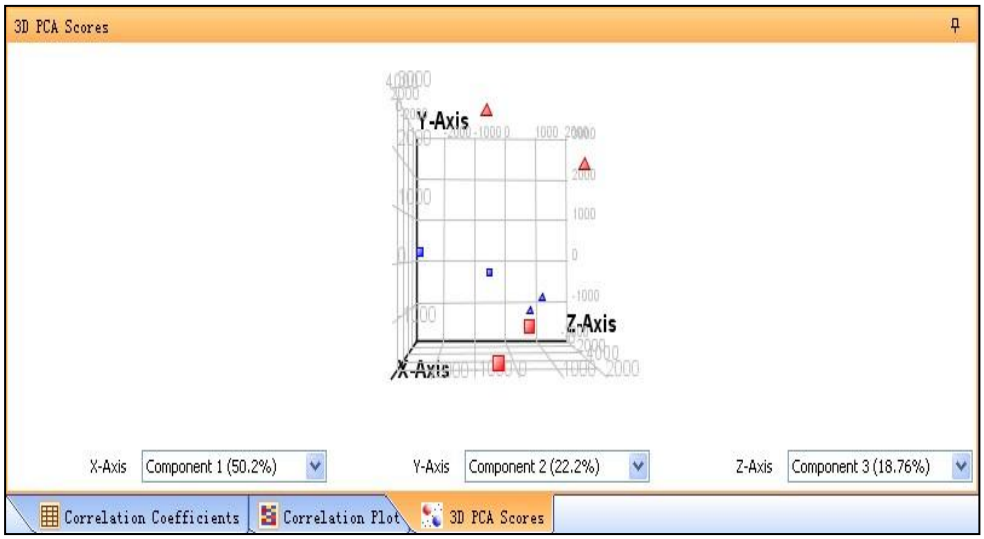


Figure 9. The PCA plot of all 8 samples in GeneSpring GX 11. 0. 2

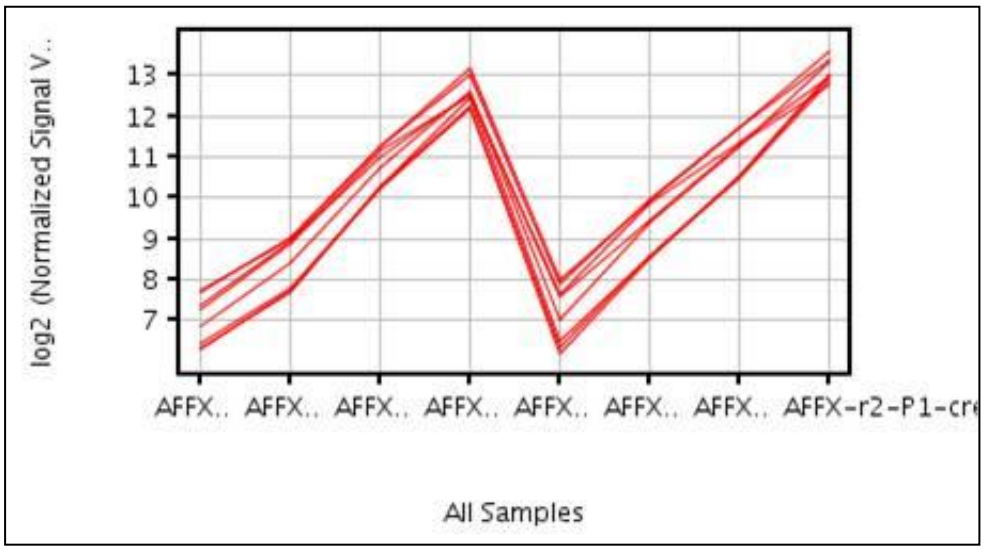


Figure 10. The Hybridization Controls of all 8 samples in GeneSpring GX 11. 0. 2

2.2.4.5 Add Technology Annotations and Import Entity lists

This part of work has been assisted by Neil Graham (NASC, The University of Nottingham). The existing annotations in GeneSpring GX 11. 0. 2 for Barley Genome are quite limited and with these annotations it's not possible to give a comprehensive understanding of how the cold-stress related genes work. Little results had been displayed when the annotation technology is only set as default as GeneSpring. Affymetrix. GeneChip. Barley1.

In this case, annotations must be added from other sources.

The annotations of *Arabidopsis thaliana* have been chosen to update the annotations of GeneSpring. Affymetrix. GeneChip. Barley1.

Table 8. Options in updating annotations from *Arabidopsis thaliana* Genome Database to GeneSpring. Affymetrix. GeneChip. Barley1 using GeneSpring GX 11. 0. 2.

Choose technology	GeneSpring. Affymetrix. GeneChip. Barley1.	
Choose source	Update from file	
Format Options	Separator	Tab
	Text qualifier	None
	Missing value indicator	None
	Comment indicator	None
Technology column to match	Gene_ID	
File column to match	All Probes	
Update Method	Overwrite	

The Arab Accn, Arab E-Score and Arab Desc were chose to add in this updating as in the following figure:

	#	Column Name	Data Type	Attribute Type	Column Mark
☐	0	Probe Set Name Found	string	Categorical	None
☐	1	Exemplar Assembly	integer	Continuous	None
☐	2	Exemplar Unigene#	integer	Continuous	None
☐	3	Pre-PolyA Trim Length	integer	Continuous	None
☐	4	Members	integer	Continuous	None
☐	5	Num. Unigenes	integer	Continuous	None
☐	6	Unigenes Represented	string	Categorical	None
☐	7	Rice Accn	string	Categorical	None
☐	8	Rice E-Score	float	Continuous	None
☐	9	Rice Chr	integer	Continuous	None
☐	10	Rice 5'	integer	Continuous	None
☐	11	Rice 3'	integer	Continuous	None
☐	12	Rice Desc	string	Categorical	None
☑	13	Arab Accn	string	Categorical	newMark1
☑	14	Arab E-Score	float	Continuous	newMark2
☐	15	Arab Chr	string	Categorical	None
☐	16	Arab 5'	integer	Continuous	None
☐	17	Arab 3'	integer	Continuous	None
☑	18	Arab Desc	string	Categorical	newMark3

Figure 11. The columns to be added in the updating annotations from *Arabidopsis thaliana* to GeneSpring. Affymetrix. GeneChip. Barley1. using GeneSpring GX 11. 0. 2.

After these steps, new annotations have been added into GeneSpring. Affymetrix. GeneChip. Barley1 successfully. With these new annotations, the entity lists can be renewed with AGI codes.

2.2.4.6 Export Entity Lists

All the entity lists should be exported and then improted into new experiment for future analysis. For all the entity lists, AGI should be selected as the annotation items and the entity list data should be selected as the data columns. All the entity lists should be exported and saved as .txt files for future import.

2.2.4.7 Set up AtGenRoot_Shoot Experiment and Import Entity lists

For Go analysis and Pathway analysis, all the exported entity lists should be imported into an experiment with the annotation technology is based on Affymetrix. GeneChip. ATH1 - 121501 to recognize all the AGI codes.

This Affymetrix. GeneChip. ATH1 – 121501 annotation technology based experiment was kindly provided by Neil Graham (NASC, The University of Nottingham). And this experiment was named as AtGenRoot_Shoot.

Using Import Entity List from Files in the Utilities block of GeneSpring GX 11. 0. 2 to import all the entity lists, only when a column in the file is matched with the identifier column in the technology will other columns from the file be available for import.
The options are listed as below:

File column to match	AGI
Technology column to match	AGI

All the imported entity lists were created from Significance Analysis with Filtered on Expression (20.0 - 100.0) the Percentile in the Raw Data for both tissue and temperature. Set the p-value cut-off as 0.05 and the fold change cut-off as 2.0 by T Test unpaired test with the p-value computation as Asymptotic and Multiple Testing Correction as No Correction.

After all these steps, it was possible to analysis the data of all 8 samples in AtGenRoot_Shoot experiment with updating annotations. The results of GO analysis and Pathway analysis could be obtained.

3 RESULTS

3.1 Expression profiles of cold regulated genes in root and leaf

To identify genes of potential importance to cold, global expression profiling was measured in barley plants subjected to cold stress (4 ℃) using barley Affymetrix GeneChip. Total RNA was prepared from barley seedlings (root and leaf tissues). Roots were exposed to cold stress (4 ℃) and control plants grown at 20 ℃. The Affymetrix array data with double replicates was analyzed for cold-regulated genes that showed a greater than 2-fold change in expression compared with control plants. The 8 samples have been divided into 3 groups as conditions to be compared.

Table 9. conditions to be compared by Filter on Volcano Plot to find genes and related pathways of interest using GeneSpring GX 11. 0. 2

Group	**Conditon 1**	**Condition 2**
1	[root, 4℃]	[root, 20℃]
2	[root, 4℃]	[leaf, 20℃](with root as 4℃)
3	[leaf, 20℃] (with root as 4℃)	[leaf, 20℃]

For group one **[root, 4 ℃] Vs [root, 20 ℃]**, there are **682** entities out of 19925 statisfying p-value cut-off 0.05 and fold change cut-off 2.0 as **down** regulated genes and **294** entities out of 19925 statisfying p-value cut-off 0.05 and fold change cut-off 2.0. as **up** regulated genes (Fig 20 and Fig 21).

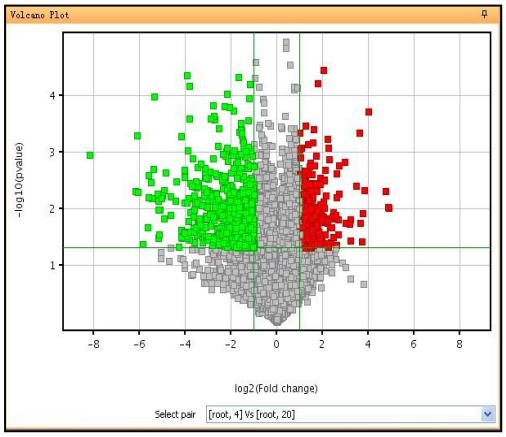


Figure 12. The Volcano Plot of all the down regulated genes in comparison of [root, 4℃] Vs [root, 20℃] with p-value cut-off as 0.05 and fold change cut-off as 2.0 with the Annotation Technology as GeneSpring. GeneLevel. Affymetrix_ GeneChip _Barley1 using GeneSpring GX 11. 0. 2

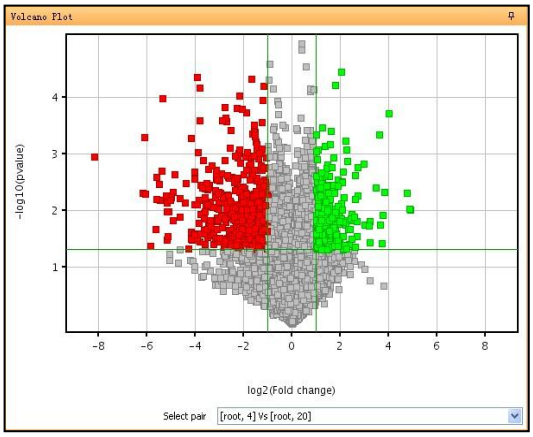


Figure 13. The Volcano Plot of all the up regulated genes in comparison of [root, 4℃] Vs [root, 20℃] with p-value cut-off as 0.05 and fold change cut-off as 2.0 with the Annotation Technology as GeneSpring. GeneLevel. Affymetrix_ GeneChip _Barley1 using GeneSpring GX 11. 0. 2

For group 2 **[root, 4 ℃] Vs [leaf, 20 ℃](with root as 4 ℃)**, there are **2074** entities out of 19925 statisfying p-value cut-off 0.05 and fold change cut-off 2.0 as **down** regulated genes and **2361** entities out of 19925 statisfying p-value cut-off 0.05 and fold change cut-off 2.0 as **up** regulated genes.

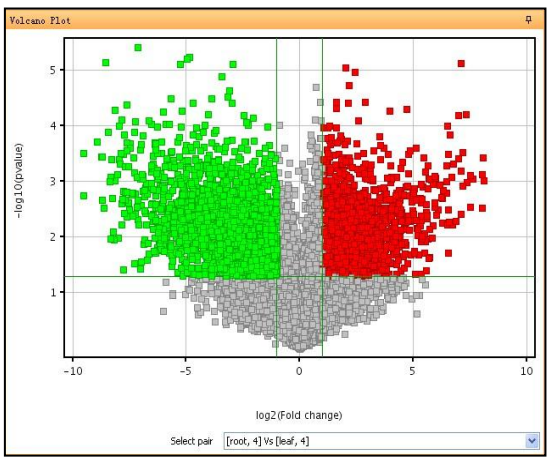


Figure 14. The Volcano Plot of all the down regulated genes in comparison of group 2 with p-value cut-off as 0.05 and fold change cut-off as 2.0 with the Annotation Technology as GeneSpring. GeneLevel. Affymetrix_ GeneChip_ Barley1 using GeneSpring GX 11. 0. 2

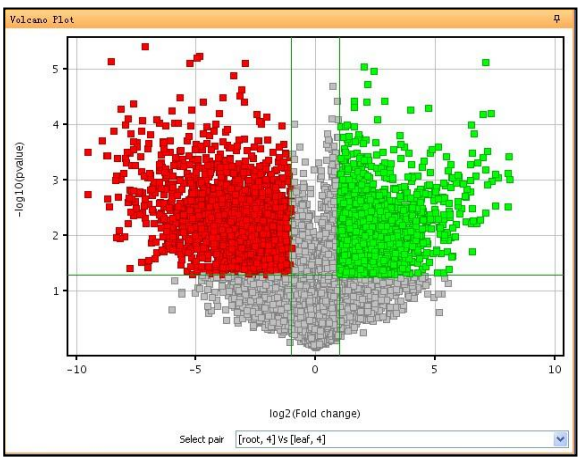


Figure 15. The Volcano Plot of all the up regulated genes in comparison of group 2 with p-value cut-off as 0.05 and fold change cut-off as 2.0 with the Annotation Technology as GeneSpring. GeneLevel. Affymetrix_ GeneChip_ Barley1 using GeneSpring GX 11. 0. 2

For group three **[leaf, 20 ℃] (with root as 4 ℃) Vs [leaf, 20 ℃]**, there are **1438** entities out of 19925 statisfying p-value cut-off 0.05 and fold change cut-off 2.0 as **down** regulated genes and **1139** entities out of 19925 statisfying p-value cut-off 0.05 and fold change cut-off 2.0 as **up** regulated genes.

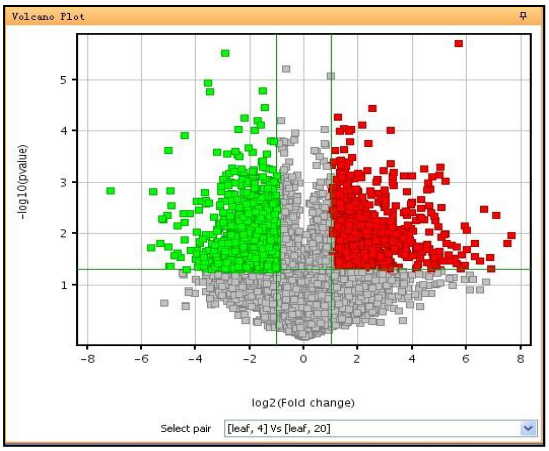


Figure 16. The Volcano Plot of all the down regulated genes in comparison of group 3 with p-value cut-off as 0.05 and fold change cut-off as 2.0 with the Annotation Technology as GeneSpring. GeneLevel. Affymetrix_ GeneChip _ Barley1 using GeneSpring GX 11. 0. 2

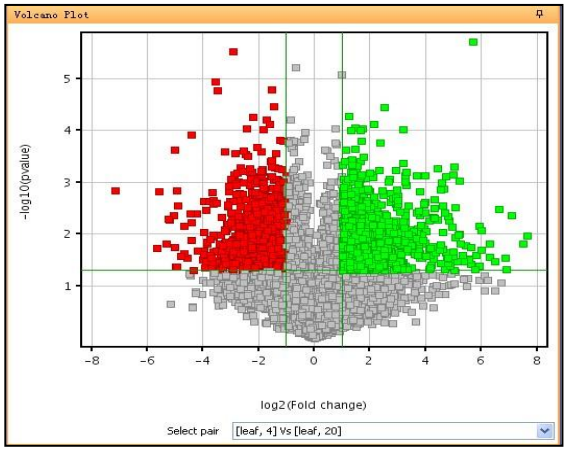


Figure 17. The Volcano Plot of all the up regulated genes in comparison of group 3 with p-value cut-off as 0.05 and fold change cut-off as 2.0 with the Annotation Technology as GeneSpring. GeneLevel. Affymetrix _ GeneChip _ Barley1 using GeneSpring GX 11. 0. 2

3.2 GO Analysis

GO analysis and Pathway analysis provided by GeneSpring GX 11. 0. 2 are the principal methods to get results interpreations.

3.2.1 Pie Charts of Overall Output Views

For group one **[root, 4 ℃] Vs [root, 20 ℃]**, the p-value was set as 1.0 and 1818 GO terms were calculated to satisfy this p-value. This pie chart (Fig.26) gives a general output view of all the GO terms that satisfying corrected p-value cut-off as 1.0. 407

GO terms are related to cellular component, 392 GO terms are related to biological process while 414 GO terms were related to molecular function.

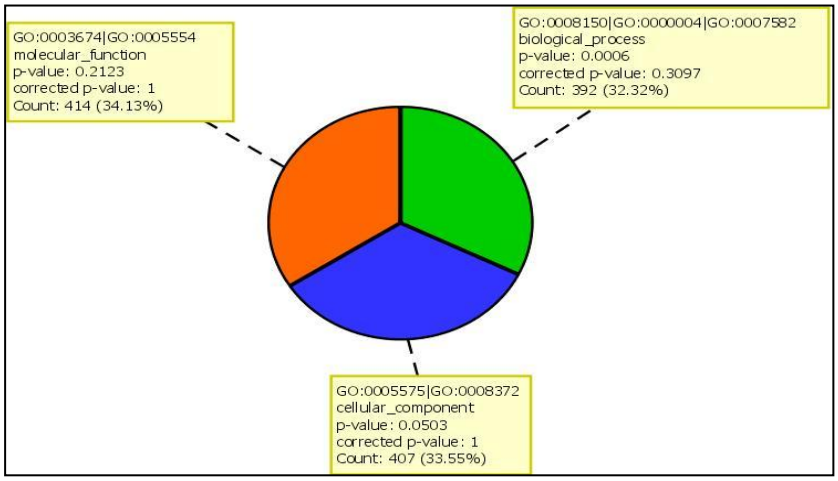


Figure 18. Overall Output View Pie Chart of group 1 got from GO Analysis using GeneSpring GX 11. 0. 2

For group two **[root, 4 ℃] Vs [leaf, 20 ℃](with root as 4 ℃)**, the p-value was set as 1.0 and 3438 GO terms were calculated to satisfy this p-value. This pie chart (Fig. 27) gives a general output view of all the GO terms that satisfying corrected p-value cut-off as 1.0. 2028 GO terms are related to cellular component, 1777 GO terms are related to biological process while 1888 GO terms were related to molecular function.

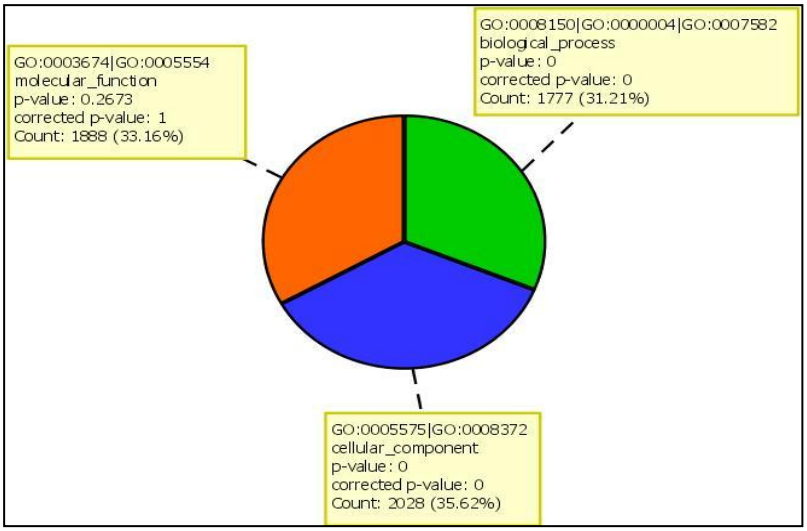


Figure 19. Overall Output View Pie Chart of group 2 got from GO Analysis using GeneSpring

GX 11. 0. 2

For group three **[leaf, 20 ℃] (with root as 4 ℃) Vs [leaf, 20 ℃]**, the p-value was set as 1.0 and 2986 GO terms were calculated to satisfy this p-value. This pie chart (Fig. 28) gives a general output view of all the GO terms that satisfying corrected p-value cut-off as 1.0. 1213 GO terms are related to cellular component, 1086 GO terms are related to biological process while 1141 GO terms were related to molecular function.

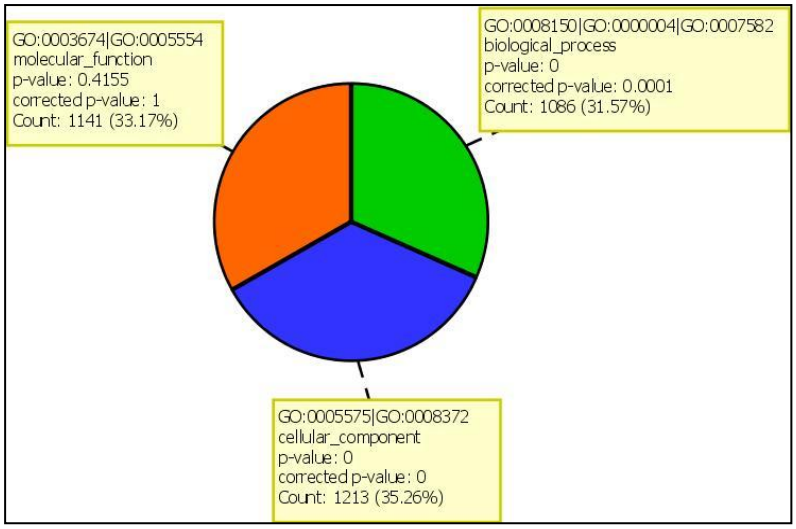


Figure 20. Overall Output View Pie Chart of group 3 got from GO Analysis using GeneSpring GX 11. 0. 2

3.2.2 Cold-Stress Response Related GO Terms

For all these three groups, the GO Analysis parameters corrected p-value cut-off was set as 1.0 and the Genes were selected from GO Tree.

For group 1 **[root, 4 ℃] Vs [root, 20 ℃]**, there are 5 cold-stress response genes were calculated from GO Tree.

Table 10. 5 Genes that related to cold-stress response of group 1 calculated from GO Tree with the adjusted p value as 1.0 and the p value as 0.006 and the Annotation Technology as Affymetrix. GeneChip. ATH-121501 using GeneSpring GX 11. 0. 2

Probe Set ID	AGI	Gene Symbol

262784_at	AT1G10760	SEX1
256417_s_at	AT3G11170/ AT5G05580	FAD7
254286_at	AT4G22950	AGL19
251625_at	AT3G57260	BGL2
247095_at	AT5G66400	RAB18

There are 2 Genes in this GO group that related to Cold Acclimation.

Table 11. 2 Genes that related to cold acclimation of group 1 calculated from GO Tree with the adjusted p value as 0.16 and the p value as 2.826E-4 and the Annotation Technology as Affymetrix. GeneChip. ATH-121501 using GeneSpring GX 11. 0. 2

Probe Set ID	**AGI**	**Gene Symbol**
262784_at	AT1G10760	SEX1
247095_at	AT5G66400	RAB18

There is 1 Gene in this GO group that related to Vernalization Response.

Table 12. One Gene that related to vernalization response of group 1 calculated from GO Tree with the adjusted p value as 1.0 and the p value as 0.265 and the Annotation Technology as Affymetrix. GeneChip. ATH-121501 using GeneSpring GX 11. 0. 2

Probe Set ID	**AGI**	**Gene Symbol**
254286_at	AT4G22950	AGL19

For group 2 **[root, 4 ℃] Vs [leaf, 20 ℃](with root as 4 ℃)**, there are 10 cold-stress response genes calculated from GO Tree.

Table 13. 10 Genes that related to cold-stress response of group 2 calculated from GO Tree with the adjusted p value as 2.697E-7 and the p value as 8.184E and the Annotation Technology as Affymetrix. GeneChip. ATH-121501 using GeneSpring GX 11. 0. 2

Probe Set ID	**AGI**	**Gene Symbol**
262064_at	AT1G56070	LOS1

261899_at	AT1G80820	CCR2
261792_at	AT1G15950	CCR1
259625_at	AT1G42970	GAPB
259570_at	AT1G20440	COR47
258310_at	AT3G26744	ICE1
256417_s_at	AT3G11170 /AT5G05580	FAD7
247523_at	AT5G61410	RPE
247095_at	AT5G66400	RAB18
245251_at	AT4G17615	CBL1

There are 2 Genes in this GO group that related to Cold Acclimation.

Table 14. 2 Genes that related to cold acclimation of group 2 calculated from GO Tree with the adjusted p value as 1.0 and the p value as 0.14 and the Annotation Technology as Affymetrix. GeneChip. ATH-121501 using GeneSpring GX 11. 0. 2

Probe Set ID	**AGI**	**Gene Symbol**
259570_at	AT1G20440	COR47
247095_at	AT5G66400	RAB18

There is one Gene in this GO group that response to freezing.

Table 15. One Gene that response to freezing of group 2 calculated from GO Tree with the adjusted p value as 1.0 and the p value as 0.371 and the Annotation Technology as Affymetrix. GeneChip. ATH-121501 using GeneSpring GX 11. 0. 2

Probe Set ID	**AGI**	**Gene Symbol**
258310_at	AT3G26744	ICE1

For group 3 **[leaf, 20 ℃] (with root as 4 ℃) Vs [leaf, 20 ℃]**, there are 8 cold stress response genes calculated from GO Tree.

Table 16. 8 Genes that related to cold-stress response of group 3 calculated from GO Tree with the adjusted p value as 2.253E-4 and the p value as 7.912E-7 and the Annotation Technology as Affymetrix. GeneChip. ATH-121501 using GeneSpring GX 11. 0. 2

Probe Set ID	AGI	Gene Symbol
263676_at	AT1G09340	CRB
261792_at	AT1G15950	CCR1
256417_s_at	AT3G11170 /AT5G05580	FAD7
252957_at	AT4G38680	GRP2
252102_at	AT3G50970	LTI30
251905_at	AT1G42970/AT3G53710	GAPB
247523_at	AT5G61410	RPE
247095_at	AT5G66400	RAB18

There are 2 Genes in this GO group that related to Cold Acclimation.

Table 17. 2 Genes that related to cold acclimation of group 3 calculated from GO Tree with the adjusted p value as 1.0 and the p value as 0.085 and the Annotation Technology as Affymetrix. GeneChip. ATH-121501 using GeneSpring GX 11. 0. 2

Probe Set ID	AGI	Gene Symbol
252102_at	AT3G50970	LTI30
247095_at	AT5G66400	RAB18

3.2.3 Summary of GO Analysis

There are many genes that have been found related to Cold Stress Response through different GO groups.

For group one **[root, 4 ℃] Vs [root, 20 ℃]**, there are three GO groups involved:

1. Response to Cold
2. Cold Acclimation,

3. Vernalization Response.

There are 3 genes that may of interest according to comparison of Table 10, 11, 12.

Table 18. 3 genes that may of interest according to comparison of Table 10, 11, and 12 in group 1 by using GeneSpring GX 11. 0. 2

Gene Symbol	Probe Set ID	Type	Entrez ID	AGI	Regulation
SEX1	Contig18313_at	Protein	837619	AT1G10760	Up
FAD7	Contig7663_at	Protein	820288	AT5G05580	Up
RAB18	Contig1709_at	Protein	836772	AT5G66400	Up

For group two **[root, 4 ℃] Vs [leaf, 20 ℃](with root as 4 ℃)**, there are three GO groups involved:

1. Response to Cold
2. Cold Acclimation
3. Response to Freezing

There are 2 genes that may of interest according to comparison of Table 13, 14, 15.

Table 19. 2 genes that may of interest according to comparison of Table 13, 14, and 15 in group2 by using GeneSpring GX 11. 0. 2

Gene Symbol	Probe Set ID	Type	Entrez ID	AGI	Regulation
ICE1	Contig13678_at	Protein	822287	AT3G26744	Down
RAB18	Contig1713_s_at	Protein	836772	AT5G66400	Down

For group three **[leaf, 20 ℃] (with root as 4 ℃) Vs [leaf, 20 ℃]**, there are two GO groups involved:

1. Response to Cold
2. Cold Acclimation

There are 4 genes that may of interest according to comparison of Table 16, 17.

Table 20. 4 genes that may of interest according to comparison of Table 16, 17 in group 3 by using GeneSpring GX 11. 0. 2

Gene Symbol	Probe Set ID	Type	Entrez ID	AGI	Regulation
SEX1	Contig18313_at	Protein	837619	AT1G10760	Up
FAD7	Contig7663_at	Protein	820288	AT5G05580	Up
RAB18	Contig1709_at	Protein	836772	AT5G66400	Up
LTI30	Contig1717_s_at	Protein	824261	AT3G50970	Up

In the subsequently Pathway Analysis, these genes could be focus on to find their correlations with other genes and biological processes.

3.3 Pathway Analysis

GeneSpring GX 11. 0. 2 provide two Analysis Types and corresponding Algorithms for Pathway Analysis. All the entity lists that saved from GO analysis have been inputted through all these Algorithms to get the final results about cold-stress response Genes and the most relevant regulators.

3.3.1 Group One: [root, 4 ℃] Vs [root, 20 ℃]

3.3.1.1 Response to Cold

3.3.1.1.1 Expand Interactions Pathway

Set the Analysis Type as Advanced and the Algorithm as Expand Interactions, Relation score was chose as >=1, the Entity local connectivity as >=1, all relation type and entity type were selected, then the Pathway Analysis Plot was output as below:

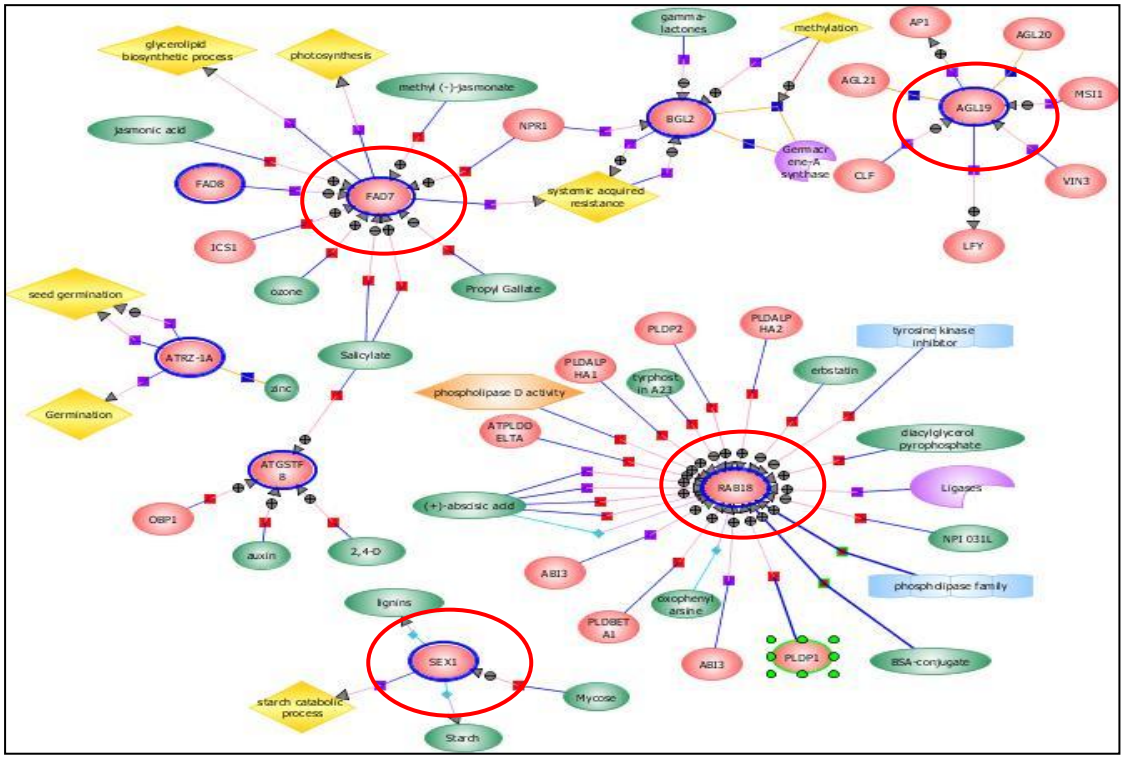


Figure 21. Expand Interaction Pathway Output View of GO group response to cold in group 1 with Relation score was chose as >=1, the Entity local connectivity as >=1, all relation type and entity type were selected using GeneSpring GX 11. 0. 2

- There are 8 genes that related to cold stress response which are circled with blue revealing that their expressions in this pathway are significant. The four genes that selected from GO group which may of interest are circled with red.
- RAB18 is shown as the major target of local relevant connectivities both on expression level and regulation level. RAB18 doesn't regulate any other genes while it is controlled by ABA. RAB18 is up regulated by ABI3, PLDBETA1, AB13, PLDP1, PLDP2, PLDALPHA2, ATPLDDELTA. As a result, the accumulation of RAB18 is quite high in group one.
- FAD7 is another major target of local relevant connectivities. FAD7 is down regulated by FAD8 (FATTY ACID DESATURASE 8) which is also a member of FAD protein family. FAD7 is up regulated by jasmonic acid, zone and some other proteins which didn't found to be related to cold stress response.
- SEX1 is identified to participate in the starch catabolic process and is depressed by Mycose. It's also a very important reactant in the production of Lignins and Starch.

3.3.1.1.2 Other Pathways

Network Targets and Regulators Pathway:

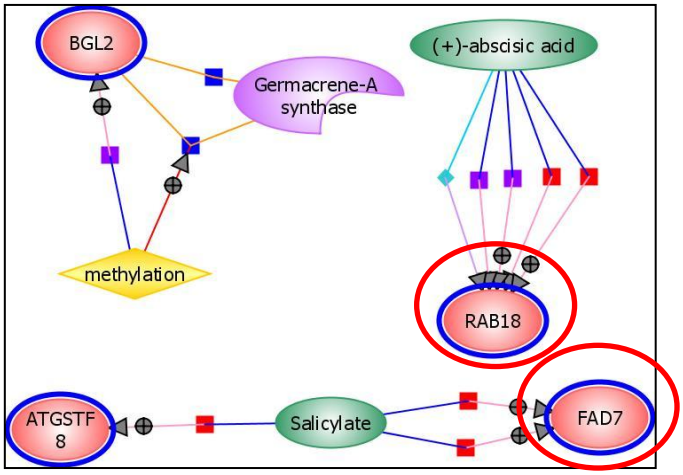


Figure 22. Network Targets and Regulators Pathway Output View of GO group response to cold in group 1 by using GeneSpring GX 11. 0. 2

There are 4 genes that were found as main network targets and regulators in this pathway.

Table 21. 4 genes that were found as main network targets and regulators in GO group response to cold of group 1 by using GeneSpring GX 11. 0. 2

Name	Entrez ID	Regulation	Name	Entrez ID	Regulation
ATGSTF8	819386	Down	BGL2	824893	Down
FAD7	820288	Up	RAB18	836772	Up

- Among these 4 genes, only FAD7 and RAB18 were found to have significant correlation with cold stress response. RAB18 is under the control of ABA.

Transcription Regulators Pathway:

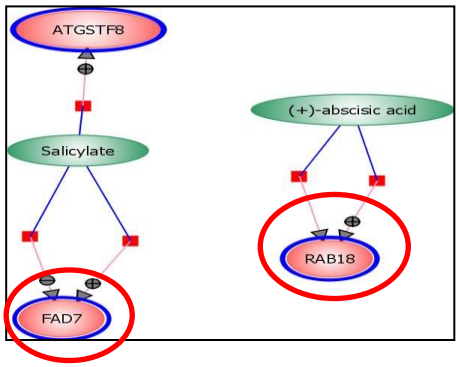


Figure 23. Transcription Regulators Pathway Output View of GO group response to cold in group 1 by using GeneSpring GX 11. 0. 2

There are 3 genes that were found as main transcription regulators in this pathway.

Table 22. 3 genes that were found as main transcription regulators in GO group response to cold in group 1 using GeneSpring GX 11. 0. 2

Name	Entrez ID	Regulation
ATGSTF8	819386	Down
FAD7	820288	Up
RAB18	836772	Up

- Among these 3 genes, only FAD7 and RAB18 were found to have significant correlation with cold stress response. And also, RAB18 was significantly up regulated by ABA while FAD7 was under the control of Salicylate.

3.3.1.2 Cold Acclimation

3.3.1.2.1 Expand Interactions

Set the Analysis Type as Advanced and the Algorithm as Expand Interactions, Relation score was chose as >=1, the Entity local connectivity as >=1, all relation type and entity type were selected, then the Pathway Analysis Plot was output as below:

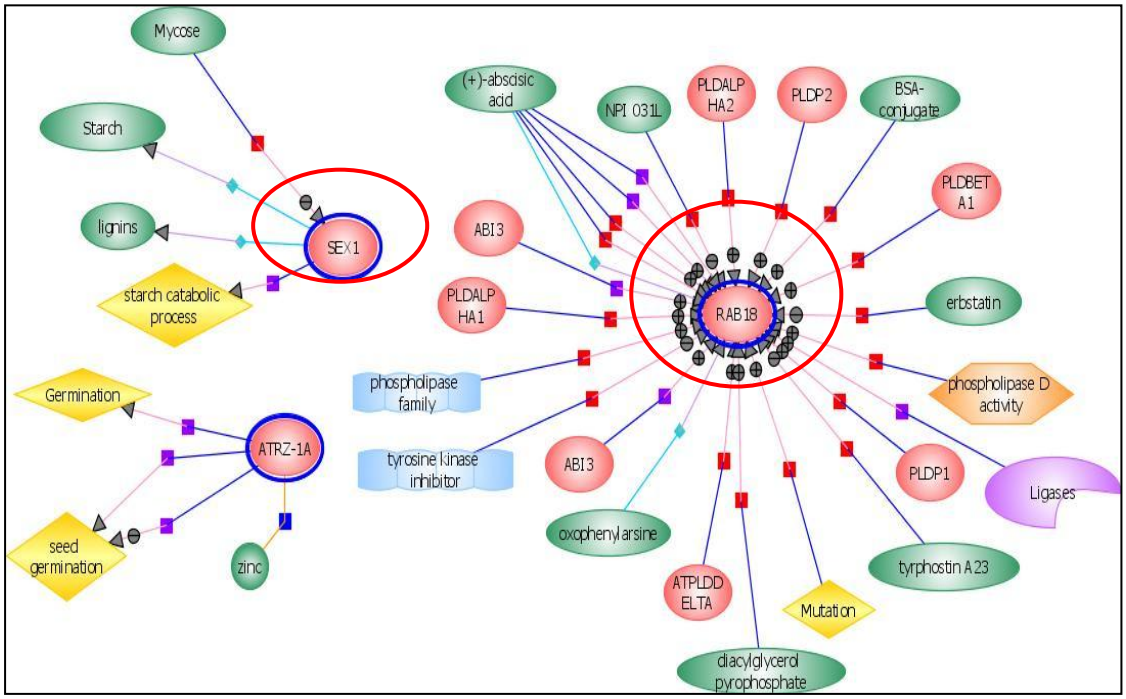


Figure 24. Expand Interaction Pathway Output View of GO group cold acclimation group 1 with Relation score was chose as >=1, the Entity local connectivity as >=1, all relation type and entity type were selected using GeneSpring GX 11. 0. 2

From this overview plot, there are 2 genes that have strong interactions with other nodes and biological processes.

Table 23. 2 genes that have strong interactions with other nodes and biological processes in Figure 24 by usingGeneSpring GX 11. 0. 2

Name	Entrez ID	Regulation
SEX1	837619	Up
RAB18	836772	Up

- There are 3 genes that related to Cold Acclimation which were circled with blue revealing that their expressions in this pathway are significant. The two genes that selected from GO group which may of interest are circled with red.
- RAB18 is shown as the major target of local relevant connectivities both on expression level and regulation level. RAB18 doesn't regulate any other genes while under the control of ABA. RAB18 is up regulated by ABI3, PLDBETA1, AB13, PLDP1, PLDP2, PLDALPHA2, ATPLDDELTA. As a result, the accumulation of RAB18 is considerable high in group one.
- SEX1 is identified to participate in the starch catabolic process and is depressed by Mycose. It's also a very important reactant in the production of Lignins and Starch.

3.3.1.2.2 Other Pathways

Network Targets and Regulators Pathway:

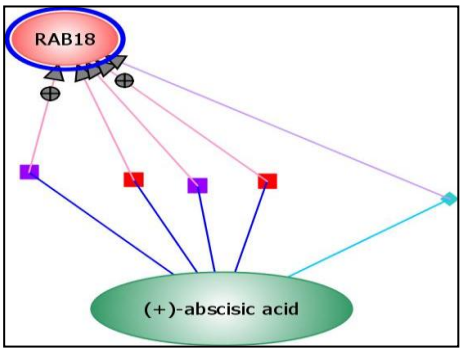


Figure 25. Network Targets and Regulators Pathway Output View of GO group cold acclimation in group 1 by using GeneSpring GX 11. 0. 2

There is 1 gene that was found as main network target in this pathway.

Table 24. One gene that was found as main network target in GO group cold acclimation of group 1 by using GeneSpring GX 11. 0. 2

Name	Entrez ID	Regulation
RAB18	836772	Up

- RAB18 is the only gene displayed in this pathway, and RAB18 is shown as up expression in this group. This may suggest that RAB18 is the major Network Target that involved in Cold Acclimation when Barley roots were treated with cold temperature. And RAB18 is strongly controlled by ABA.

Transcription Regulators Pathway:

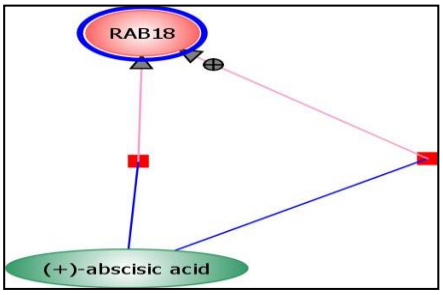


Figure 26. Transcription Regulators Pathway Output View of GO group cold acclimation in group 1 by using GeneSpring GX 11. 0. 2

There is 1 gene that was found as main transcription regulator in this pathway.

Table 25. One gene that was found as main transcription regulator of GO group cold acclimation in group 1 by using GeneSpring GX 11. 0. 2

Name	Entrez ID	Regulation
RAB18	836772	Up

- RAB18 is the only gene displayed in this pathway, and RAB18 is shown as up

expression in this group. This may suggest that RAB18 is the major Transcription Regulator that involved in Cold Acclimation when Barley roots were treated with cold temperature. And RAB18 is strongly controlled by ABA.

3.3.1.3 Vernalization Response

3.3.1.3.1 Expand Interactions

Set the Analysis Type as Advanced and the Algorithm as Expand Interactions, Relation score was chose as >=1, the Entity local connectivity as >=1, all relation type and entity type were selected, then the Pathway Analysis Plot was output as below:

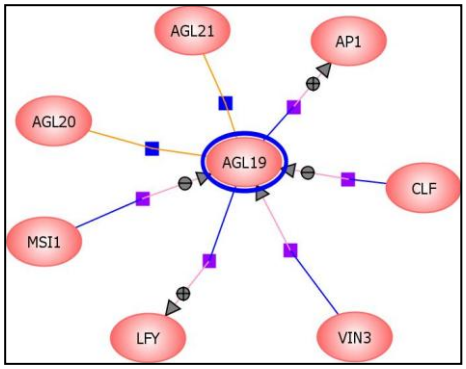


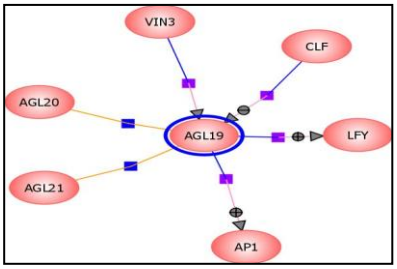
Figure 27. Expand Interaction Pathway Output View of GO group vernalization response group one [root, 4℃] Vs [root,20℃] with Relation score was chose as >=1, the Entity local connectivity as >=1, all relation type and entity type were selected using GeneSpring GX 11. 0. 2

From this overview plot, there is one gene that has strong interactions with other nodes.

Table 26. One gene that has strong interactions with other nodes in Figure 27 by using GeneSpring GX 11. 0. 2

Name	Entrez ID	Regulation
AGL19	828394	Up

3.2.1.3.2 Network Targets and Regulators Pathway



There is one gene that was found as the main network target and regulator in this pathway.

Table 27. One gene that was found as main network target and regulator in GO group vernalization response of group 1 by using GeneSpring GX 11. 0. 2

Name	Entrez ID	Regulation
AGL19	828394	Down

- AGL19 is the only gene that found in Vernaliztion Response pathway. This may suggest that AGL19 is the major network regulator involved in Vernalization Response. As AP1 and LFY are up regulated by AGL19, it's most likely to promote the flowering of plants when the roots were treated with low temperature. However, different from theory, AGL19 is found as down expression in this group, which worth leading to future discussion.

3.3.2 Group Two: [root, 4 ℃] Vs [leaf, 20 ℃](with root as 4 ℃)

3.3.2.1 Response to Cold

3.3.2.1.1 Expand Interactions Pathway

Set the Analysis Type as Advanced and the Algorithm as Expand Interactions, Relation score was chose as >=1, the Entity local connectivity as >=1, all relation type and entity type were selected, then the Pathway Analysis Plot was output as below:

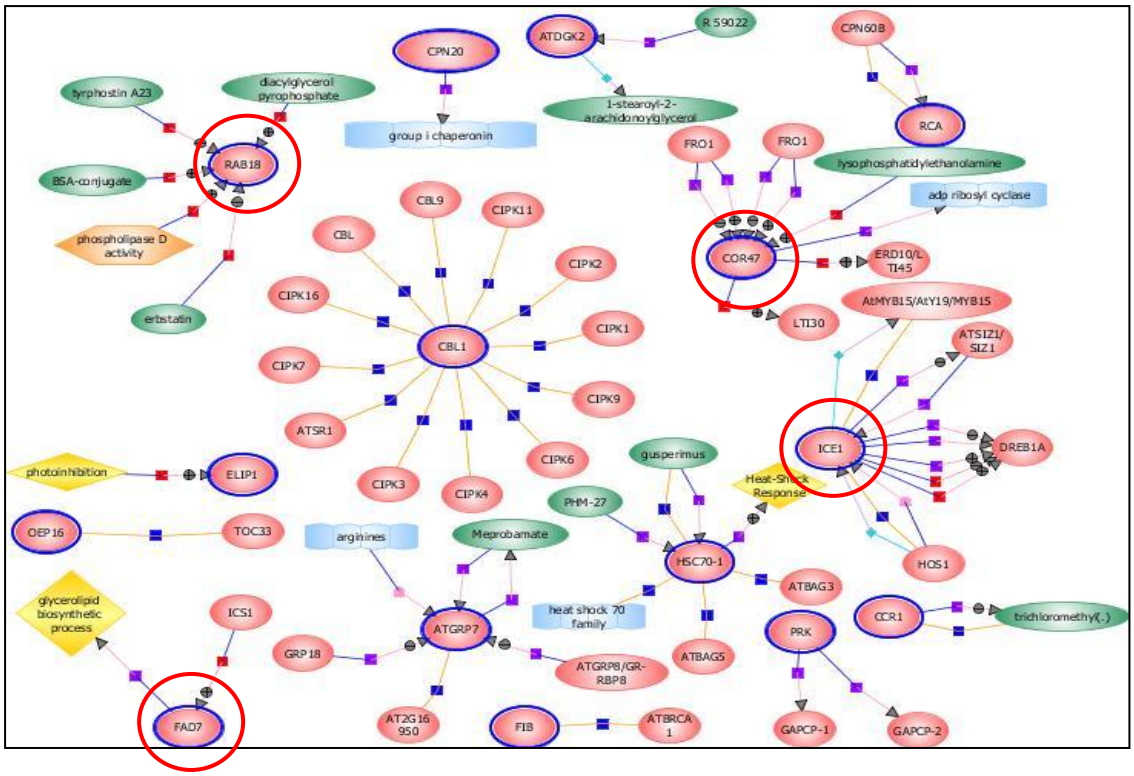


Figure 28. Expand Interaction Pathway Output View of GO group response to cold in group 2 with Relation score was chose as >=1, the Entity local connectivity as >=1, all relation type and entity type were selected using GeneSpring GX 11. 0. 2

From this overview plot, there are 15 genes that have strong interactions with other nodes and biological processes:

Table 28. 15 genes that have strong interactions with other nodes and biological processes in Figure 28 by usingGeneSpring GX 11. 0. 2

Name	Entrez ID	Name	Entrez ID
COR47	838632	CBL1	827481
CCR1	838165	PRK	831020
OEP16	817439	HSC70-1	831020
CPN20	832195	FAD7	820288
RCA	818558	ATDGK2	836497
ATGRP7	816705	ICE1	822287
FIB	825714	RAB18	836772
ELIP1	821855		

- There are 15 genes that related to cold stress response which are circled with blue revealing that their expressions in this pathway are significant. The four genes that selected from GO group which may of interest are circled with red.
- After identifying the functions of each gene, there are 4 genes that show high coherence with Response to Cold: COR47, ICE1, FAD7, RAB18.
- COR47 (COLD REGULATED 47) responses to cold and participates in the cold acclimation. It was only found in Expand interactions Pathway, this may suggest that COR47 is not the major transcription regulator or network target during cold acclimation. However, evidence has shown that COR47 is of high correlation with RAB18. The correlation between COR47 and RAB18 will be discussed in discussion part.
- As a symbol gene of Cold Stress Response, FAD7 is shown as not significant up or down expression in this group. And there are little nodes that connected with FAD7 in this pathway.
- As a gene that response to freezing, ICE1 is found as significant down expression in this group.

3.3.2.1.2 Other Pathways

Network Targets and Regulators Pathway:

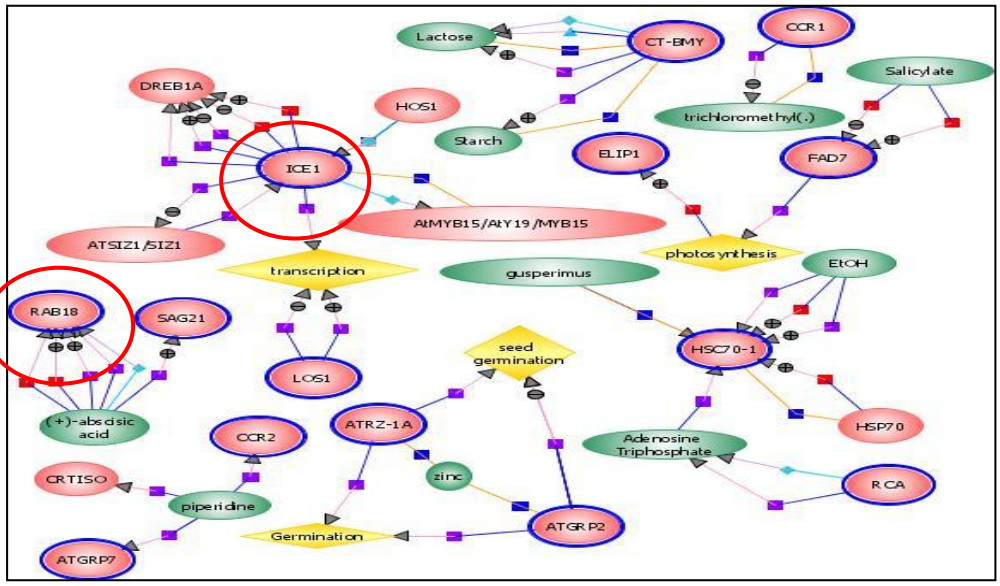


Figure 29. Network Targets and Regulators Pathway Output View of GO group response to cold in group 2 by using GeneSpring GX 11. 0. 2

There are 14 genes found as main network targets and regulators in this pathway.

Table 29. 14 genes found as main network targets and regulators in GO group cold response of group 2 by using GeneSpring GX 11. 0. 2

Name	Entrez ID	Name	Entrez ID
CCR1	838165	ATRZ-1A	822246
SAG21	828053	ELIP1	821855
ATGRP2	827019	HSC70-1	831020
RCA	818558	FAD7	820288
LOS1	842058	CCR2	844421
CT-BMY	827419	ICE1	822287
ATGRP7	816705	RAB18	836772

- There are 14 genes that related to cold stress response which are circled with blue revealing that their expressions in this pathway are significant. The two genes that selected from GO group which may of interest arecircled with red.
- After identify the function of each gene, the two genes circled with red are considered as the principal Network Targets and Regulators.
- ICE1 is found as significant down expression in this pathway. This is may be caused by the existing of HOS1 (HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1). HOS1 is a reactant in the production of ICE1. The absence of HOS1 would dramatically reduce the expression of ICE1 which resulting fall to enhance the cold tolerance of plants.
- Different from group one, RAB18 as shown as significant down expression in this group. This is perhaps due to the selective location characterization of RAB18. So the expression of RAB18 was different in tissues.

Transcription Regulators Pathway

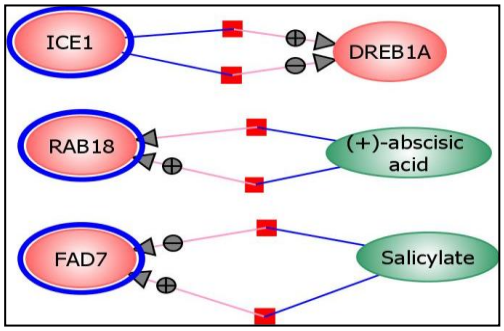


Figure 30. Transcription Regulators Pathway Output View of GO group response to cold in group 2 by using GeneSpring GX 11. 0. 2

There are 3 genes found as main Transcription Regulators in this pathway.

Table 30. 3 genes found as main transcription regulators in GO group response to cold of group 2 by using GeneSpring GX 11. 0. 2

Name	Entrez ID	Regulation

FAD7	820288	NA
ICE1	822287	Down
RAB18	836772	Down

- ICE1, RAB18, FAD7 are the three genes that found as the main Transcription Regulators in this pathway. However, FAD7 is not found as significant up or down expression. RAB18 is under control of ABA while ICE1 mainly regulates the expression of DREB1A, a gene that responses to water deprivation.

3.3.2.2 Response to Freezing

3.3.2.2.1 Expand Interactions Pathway

Set the Analysis Type as Advanced and the Algorithm as Expand Interactions, Relation score was chose as >=1, the Entity local connectivity as >=1, all relation type and entity type were selected, then the Pathway Analysis Plot was output as below:

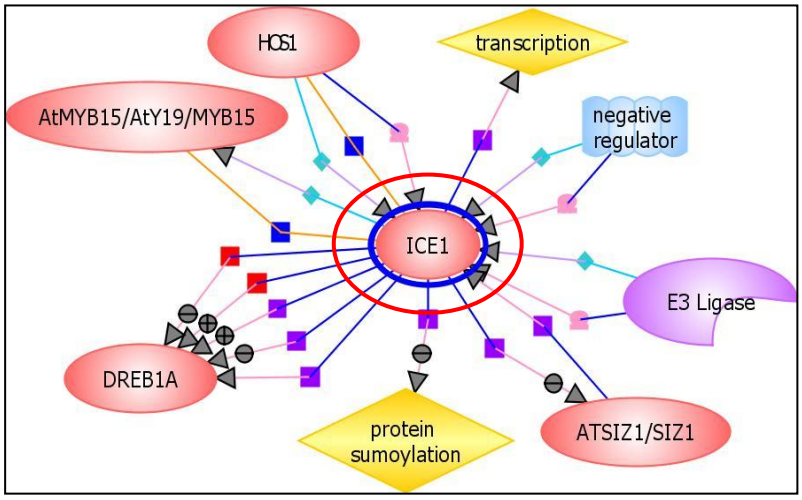


Figure 31. Expand Interactions Pathway Output View of GO group response to freezing group 2 with Relation score was chose as >=1, the Entity local connectivity as >=1, all relation type and entity type were selected using GeneSpring GX 11. 0. 2

From this overview plot, there is one gene that has strong interactions with other nodes and biological processes.

Table 31. One gene that has strong interactions with other nodes and biological processes in Figure 31 by usingGeneSpring GX 11. 0. 2

Name	Entrez ID	Regulaton
ICE1	822287	Down

3.3.2.2.2 Other Pathways

Network Targets and Regulators Pathway

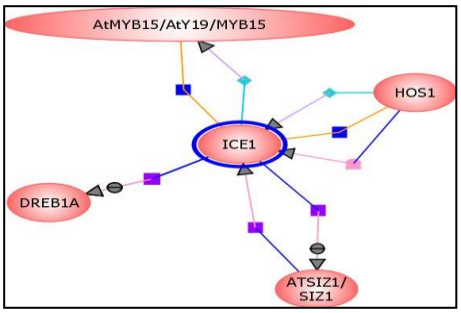


Figure 32. Network Targets and Regulators Pathway Output View of GO group response to freezing in group 2 by using GeneSpring GX 11. 0. 2

There is one gene that found as main network target and regulator in this pathway.

Table 32. One gene that found as main network target and regulator in GO group response to freezing of group 2 using GeneSpring GX 11. 0. 2

Name	Entrez ID	Regulation
ICE1	822287	Down

- ICE1 is the main regulator in these pathways which illustrate that ICE1 is of central important in the response to freezing in plants. ICE1 can enhance the cold and freezing tolerance of plants to a certain extent which can help plants to cope with cold temperature.
- After indentify the function of each gene, DERB1A is found to have high correlation with cold stress response. DREB1A is the main regulation target of ICE1. DREB1A is involved in response to low temperature as one of the member

of the DREB subfamily A-1 of CBF3 (ERF/AP2 transcription factor family) which can be encoded by DREB1A (Novillo F et al., 2007). This may suggest that when Barley plants were treated with cold, ICE1 regulated DREB1A which can encode the family member of CBF3 and eventually enhance the cold tolerance in plants.

3.3.3 Group Three: [leaf, 20℃](with root as 4℃) Vs [leaf, 20℃]

3.3.3.1 Response to Cold

3.3.3.1.1 Expand Interactions Pathway

Set the Analysis Type as Advanced and the Algorithm as Expand Interactions, Relation score was chose as >=1, the Entity local connectivity as >=1, all relation type and entity type were selected, then the Pathway Analysis Plot was output as below:

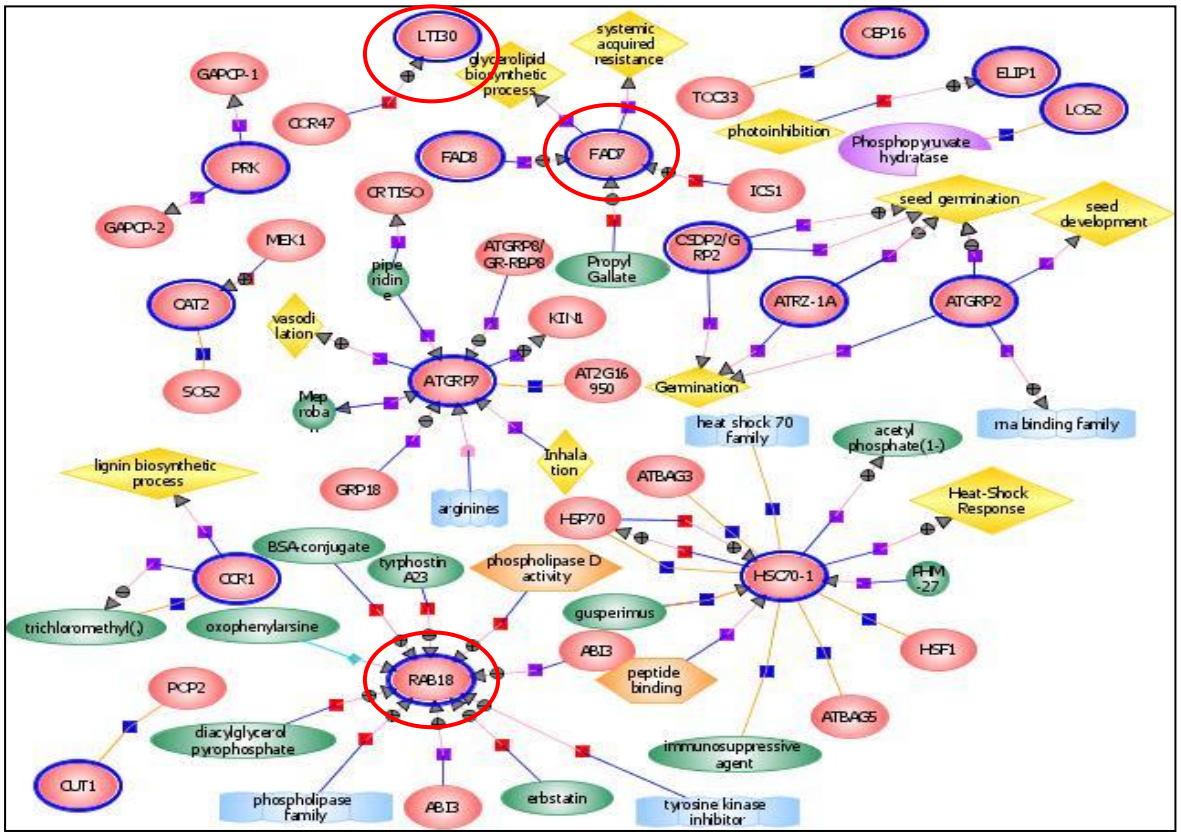


Figure 33. Expand Interaction Pathway Output View of GO group response to cold in group 3 with Relation score was chose as >=1, the Entity local connectivity as >=1, all relation type and entity type were selected using GeneSpring GX 11. 0. 2

From this overview plot, there are 15 genes that have strong interactions with other nodes and biological processes.

Table 33. 15 genes that have strong interactions with other nodes and biological processes in Figure 33 by usingGeneSpring GX 11. 0. 2

Name	Entrez ID	Name	Entrez ID
OEP16	817439	PRK	840098
CCR1	838165	FAD7	820288
ATGRP2	827019	CAT2	829661
ATRZ-1A	822246	HSC70-1	831020
FAD8	830441	LOS2	818226
CSDP2/GRP2	830024	RAB18	836772
LTI30	824261	CUT1	843182
ELIP1	821855		

- There are 15 genes that related to cold stress response which are circled with blue revealing that their expressions in this pathway are significant. The three genes that selected from GO group which may of interest are circled with red.
- After identifying the functions of each gene, there are 4 genes that show high coherence with Response to Cold: COR47, ICE1, FAD7, RAB18.
- RAB18 is shown as significant up expression in this group. Though COR47 is not interacting directly with RAB18, it up regulates the expression of LTI30 whose expression has been proven to be engineered by RAB18.
- COR47 (COLD REGULATED 47) responses to cold and participates in the cold acclimation. It is only found in Expand interactions Pathway, this may suggest that COR47 is not the major transcription regulator or network target during cold acclimation. However, evidence has shown that COR47 is of high correlation with RAB18. The correlation between COR47 and RAB18 will be discussed in discussion part.

- As a symbol gene of cold stress response, LTI30 is only found in the pathway of group three. LTI30 and COR47 are all belong to DHN protein family and they are more sensitive to cold stress than other members. LTI30 is located at the membrane and can help plants to stand with low temperature.

3.3.3.1.2 Other Pathways

Network Targets and Regulators Pathway

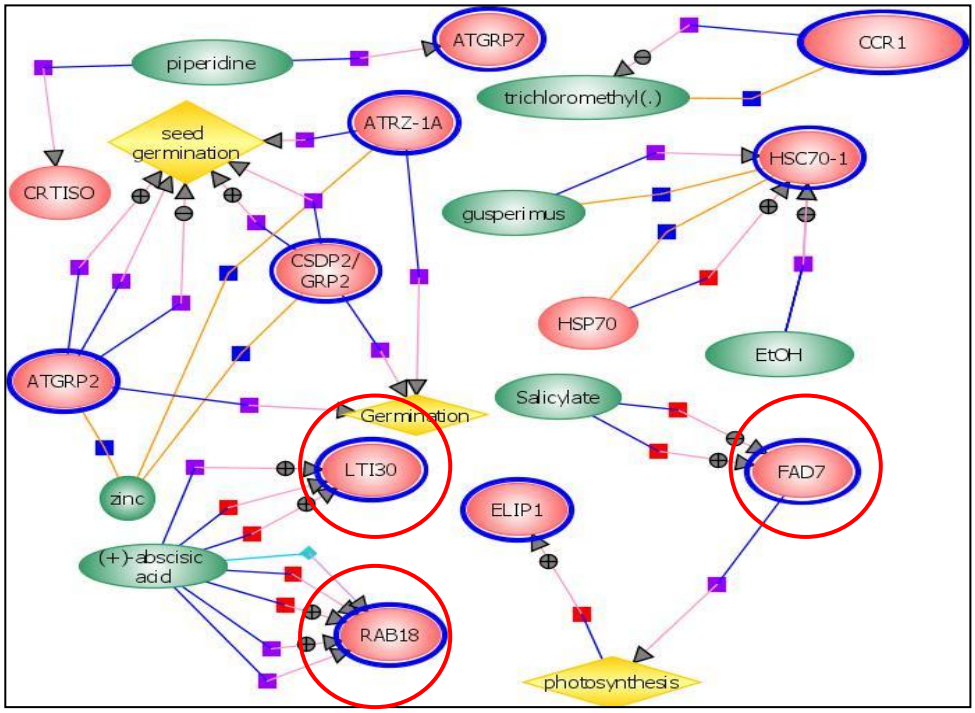


Figure 34. Network Targets and Regulators Pathway Output View of GO group response to cold in group 3by using GeneSpring GX 11. 0. 2

Transcription Regulators Pathway

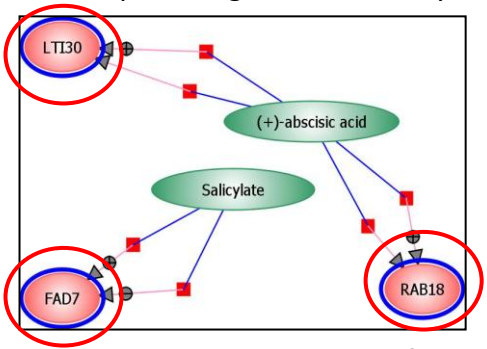


Figure 43. Transcription Regulators Pathway Output View of GO group response to cold in group 3 by using GeneSpring GX 11. 0. 2

There are 3 genes found as main Transcription Regulators in these pathways.

Table 34. 3 genes that found as main transcription regulators in GO group response to cold of group 3 by using GeneSpring GX 11. 0. 2

Name	Entrez ID	Regulation
FAD7	820288	Down
RAB18	836772	Up
LTI30	824261	Up

- FAD7, RAB18 and LTI30 are found as main transcription regulators from these pathways.
- FAD7 is shown as down expression in this group. Its function could be considered as defective to realize the protection of cold.
- RAB18 and LTI30 are both shown as up expression in these pathways. The correlation of these two genes together with COR47 will be discussed in discussion part. The up expression of both RAB18 and LTI30 may suggest that Barley can cope with cold temperature to some extent.

3.3.3.2 Cold Acclimation

3.3.3.2.1 Expand Interactions Pathway

Set the Analysis Type as Advanced and the Algorithm as Expand Interactions, Relation score was chose as >=1, the Entity local connectivity as >=1, all relation type and entity type were selected, then the Pathway Analysis Plot was output as below:

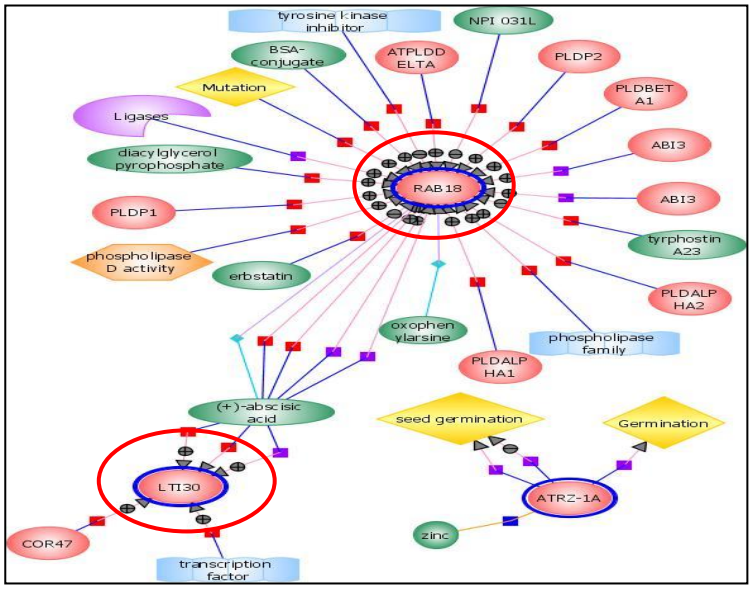


Figure 35. Expand Interactions Pathway Output View of GO group cold acclimation group 3 with Relation score was chose as >=1, the Entity local connectivity as >=1, all relation type and entity type were selected using GeneSpring GX 11. 0. 2

From this overview plot, there are 3 genes that have strong interactions with other nodes and biological processes.

Table 35. 3 genes that have strong interactions with other nodes and biological processes in Figure 35 by usingGeneSpring GX 11. 0. 2

Name	Entrez ID	Regulation
ATRZ-1A	822246	NA
RAB18	836772	Up
LTI30	824261	Up

- RAB18 and LTI30 are both shown as up expression in this pathway. This suggests that RAB18 and LTI30 are of high activity in cold acclimation. After identifying the functions of all the genes, RAB18, LTI30 and COR47 are found to be most relevant to cold stress response.

3.3.3.2.2 Other Pathways

Network Targets and Regulators Pathway:

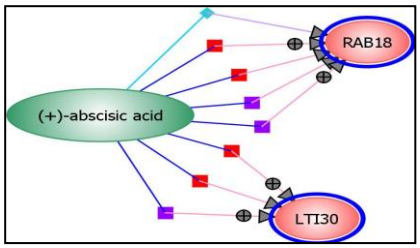


Figure 36. Network Targets and Regulators Pathway Output View of GO group cold acclimation in group 3 by using GeneSpring GX 11. 0. 2

There are two genes found as main targets and in this pathway.

Table 36. 2 genes that found as main network targets is GO group clod acclimation of group 3 by using GeneSpring GX 11. 0. 2

Name	Entrez ID	Regulation
RAB18	836772	Up
LTI30	824261	Up

Transcription Regulators Pathway:

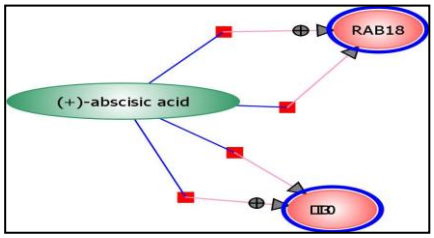


Figure 37. Transcription Regulators Pathway Output View of GO group cold acclimation in group 3 by using GeneSpring GX 11. 0. 2

There are two genes found as main transcription regulators in this pathway.

Table 37. 2 genes that found as main transcription regulators in GO group cold acclimation of group 3 by using GeneSpring GX 11. 0. 2

Name	Entrez ID	Regulation
RAB18	836772	Up
LTI30	824261	Up

- RAB18 and LTI30 are found as the only network targets and regulators in these pathways. This again suggests that these two genes are very important in cold stress response. Both of them are under control of ABA.
- How these two genes work as regulators will be discussed in discussion part.

3.4 Summary of Results

For there are a large number of genes have been found that related to cold-stress response in all kinds of pathway analysis, certain genes that have high correlation with other nodes and biological processes were picked up to get more meaningful interpretations of how Barley plants reacted to cold-stress. These genes are considered as the main regulators in the process of cold-stress response.

Firstly all these genes that selected from all kinds of Pathways were put back into the Import Lists to find out their AGI codes by searching with their Name. Secondly, the AGI codes were put back into the Export Lists to locate the regulation for each gene. The interpretations could be built up with the descriptions and regulations of these genes.

From all the pathways obtained, the genes which circled with blue demonstrated that they are the main regulation targets or regulators in cold-stress response process. From these relative genes, 6 genes that of the highest interest were picked up to explain how Barley plants may react to cold-stress on gene level (Table 38).

Table 38. 6 most relevant genes selected from aLL PathwayS of GO group response to cold in Barley using GeneSpring GX 11. 0. 2

Gene Symbol	Probe Set ID	Type	Entrez ID	AGI
AGL19	Contig26619_at	Protein	828394	AT4G22950
FAD7	Contig7663_at	Protein	820288	AT5G05580
ICE1	Contig13678_at	Protein	822287	AT3G26744
LTI30	Contig1717_s_at	Protein	824261	AT3G50970
RAB18	Contig1709_at	Protein	836772	AT5G66400
SEX1	Contig18313_at	Protein	837619	AT1G10760

These six genes are involved in different pathways and biological progresses:

AGL19 is found as the main regulate target and regulator in Vernalization Response pathway of group one [root, 4℃] Vs [root, 20℃].

ICE1 is found as the main regulate target and regulator in Response to Freezing pathway of group two [root, 4℃] Vs [leaf, 20℃](with root as 4℃).

SEX1 is found to be the main participant in starch catabolic process of group one [root, 4℃] Vs [root, 20℃.

FAD7, ITL30, RAB18, are found to have high correlations with other nodes and biological processes in all kinds of pathways.

With the discussion of these 6 genes, it's maybe possible to find out how these genes worked while the Barley plants were treated with low temperature and how their regulation relationship with other genes and biological processes.

4 DISCUSSION

4.1 Updating annotations information from *Arabidopsis thaliana* Genome Database into GebeSpring GX 11. 0. 2 for Barley GeneChip Microarray data analysis

The existed annotations for Barley microarray data analysis are very limited. Moreover there are no Pathway Analysis annotations to match the microarray data got from Barley GeneChip. To solve this problem, the annotations for data analysis were updated from *Arabidopsis thaliana* Genome Database. All the genes that have been found in experiment AtGenRoot_Shoot should be compared with the genes in original experiment to identify their regulations.

4.2 The Most Cold-Stress Response Relevant Genes in Barley plants

4.2.1 AGL19 and Vernalization Response

AGL19 is known as AGMOUS0LIKE 19 and the gene type is Protein Coding which acts as transcription factor (Elena R *et al*., 2000). AGl19 is also known as MADS-box gene AGL19 that has been identified as a novel flowering gene that is under PcG control (Schonrock N. *et al*., 2006). AGL19 has also been proven to be partly responsible for the early flowering of clf mutans (Cristina and Lars, 2008).

AGL19 was only found as significant in pathway plots of group one [root, 4℃] Vs [root, 20℃]. This is perhaps that the roots are more sensible to cold stress than leaves. Many plants need to be treated by low temperature to develop from vegetative growth to reproductive growth and Barley is one of them.

From the Expand Interactions Pathway plot, AGL19 is the participant in binding with AGL20 and AGL21 which are both highly expressed in the root. And AGL19 is down regulated by CLF. Meanwhile, AGL19 acted as the regulator and up regulated AP1 and LFY.

AP1 is floral homeotic gene specifies floral meristem and sepal identity and has interaction with LFY (Gregis v *et al*., 2009). LFY is active in floral meristem development by encoding transcriptional regulator to promote the transition to flowering (Lee J *et al*., 2008). With the increasing of AGL19 level, AP1 and LFY would eventually cause flowering (Schonrock N. *et al*., 2006).

The expression of AGL19 is under the control of PcG proteins MSI1, CLF, and EMF2 (Nicole Schonrock *et al*., 2006). If there were PcG proteins exist, the expression of AGL19 would stay at a very low level. Report has shown that AGL19 mutants have a decreased response to vernalization, and CLF can repress AGL19 in the absence of cold. That reveals prolonged cold can eliminate the repression that caused by CLF and other PcG proteins. VIN3 is the central mechanism that realizes this elimination (Nicole Schonrock *et al*., 2006).

Because the roots of Barley plants were growing at 4℃ for four weeks, this period was long enough and together with existence of VIN3 the AGL19 was believed to relieve from PcG (only CLF was found in the way)repression, and finally activated AP1 and LFY that cause flowering. Suggesting that the process of thermal induction in 4℃ treated Barley plants in which flowering was promoted by exposure to low temperatures.

But as the CLF was still down regulated AGL19 in the pathway, though AP1 and LFY were activated by AGL19, the level of AGL19 was not elevated enough to promote

the flowering of Barley Plants. And in fact no flowering process was investigated during the whole growing period.

Though the roots of Barley plants were growing at 4 degrees centigrade, the leaves were still growing at average 20 degrees centigrade which is higher than the vernalization temperature in winter.

However, as the AP1 and LFY were found up regulated by AGL19, the elimination of CLF repression can be proved. If the cold treated period was more than one mouth or the temperature was even lower, the elimination of CLF repression could be more significant.

4.2.2 FAD7 and the Protection of Cold Damage in Plants

FAD7 is known as FATTY ACID DESATURASE 7: omega-3 fatty acid desaturase.
FAD7 was up regulated according to the Filter on Volcano Plot analysis in Response to Cold pathway of group one [root, 4℃] Vs [root, 20℃]. FAD7 was indicated as significant up-regulated in three pathways in group one: Expand Interactions Pathway, Network Targets and Regulators Pathway and Transcription Regulators Pathway.

According to these pathways, FAD7 was considered as the main regulate targets. FAD7 was up regulated by ozone, ICS1, jasmonic acid, NPR1, methyl(-)-jasmonate. Salicylate acted to up regulate and down regulate FAD7 at the same time.

FAD7 was considered to be chiefly regulated by Sallicylate regarding to all the pathways that FAD7 involved in.

Among the responses to cold acclimation of plants, the associated response of increasing production of trienoic fatty fatty acid, hexadecatrienoic (16:3) and linolenic (18:3) acids is believed to protect cells against cold demage (Kodama H *et al.*,

1994). Omega-3 fatty acid desaturase gene (the FAD7 gene) is considered as the most important gene to engineer 16:3 and 18:3 fatty acids by introduction (Koh Iba *et al*., 1993).

During the cold treated growing period, there were some Barley plants began to turn yellow alongside their leaves. These parts of plants have been abandoned and the plants for doing RNA extraction all maintained as green of all the leaves. This maintaining of green may be caused by the expression of FAD7. Reports have shown that the low temperature induced chlorosis was also much reduced in the plants transformed with the FAD7 gens (Hiloaki *et al*., 1994). Researching Results indicate that increased level of trienoic fatty acids in genetically engineered plants enhance cold tolerance (Kodama H *et al*., 1994).

Because the FAD7 locus encodes a chloroplast omega-3 desaturase that accelerates the desaturation of lipid-linked 18:3 and 16:3 fatty acids (F. Martz and S. Kiviniemi), being controlled under Salicylate, FAD7 can prevent the cold-treated Barley plants from cold damage on gene expression level.

Compared with normal temperature treated group, the FAD7 was shown as up-regulated in cold temperature treated group with the reason may to strengthen the cell wall to enhance the cold tolerance physically (Khodakovskaya M *et al*., 2006).

FAD7 were also found as significant in the following pathways of group three [leaf, 20℃](with root as 4℃) Vs [leaf, 20℃] in the GO group Response to Cold: Expand Interactions Pathway, Network Targets and Regulators Pathway, Transcription Regulators Pathway.

According to the result of Filter on Volcano Plot Analysis, FAD7 was down expressed in all these pathways. As shown in the Expand Interactions Pathway, the existing of

Propyl Gallate which acted as antioxidant suppressed the expression of FAD7. Though the roots were merged into solution with temperature at 4 degrees centigrade, the leaves of same plants were growing at room temperature of average 20 degrees centigrade.

Compared with group one, FAD7 was shown down expression. For one reason that FAD7 may be down regulated by Propyl Gallate, and for another reason this may be differ from tissue growing conditions. FAD7 can protect plant cell from cold damage, this suggesting that the leaves might not be damaged by the low temperature as the roots did. Instead, the significant up expression of FAD7 in roots grew at 4 degrees centigrade demonstrated that FAD7 worked as very virtually when Barley was treated with cold to carry out protection.

To realize the protective function of FAD7, Propyl Gallate and other sorts of antioxidant should be removed or reduced to a very low level.

4.2.3 ICE1 and the Increasing Tolerance to chilling and Freezing in Plants

ICE1 is known as INDUCER OF EXPRESSION 1, it was found to be significant only in pathways of group two [root, 4℃] Vs [leaf, 20℃](with root as 4℃).

ICE1 was specific significant down expressed in the pathway of GO group Response to Freezing. ICE1 was involved in the following pathways: Expand Interactions Pathway, Network Targets and Regulators Pathway, Transcription Regulators Pathway.

ICE1 was the chief regulator of DREB1A both on expression and regulation level. And DREB1A was both down regulated and up regulated with the same possibility in this pathway. As reported has shown, DREB1A is involved in response to low temperature as one of the member of the DREB subfamily A-1 of CBF3 (ERF/AP2 transcription factor family) which can be encoded by DREB1A (Novillo F *et al*., 2007).

ICE1 is indentified as an upstream transcription factor that regulates the transcription of CBFs (C-repeat (CRT)-binding factors) genes in the cold (Chinnusamy V *et al*., 2003).

Cold temperature is a key to the expression of the CBF family to act as transcription factors. Many downstream genes could be activated by these transcription factors. And these downstream genes can increase the tolerance of chilling and freezing in plants (Viswanathan Chinnusamy *et al*., 2003).

From this point of view, when Barley plants were treated with cold temperature, ICE1 may regulate the expression of DRBE1A, and DRBE1A would encode the family member of CBF3. As been the transcription factors, CBF3 could activate the downstream genes which could allow the Barley plants to cope with low temperature by increasing the chilling and freezing tolerance (Manu Agarwal *et al*., 2006).

There is evidence show that the transcript levels of many cold-responsive genes are altered by the regulation of ICE1 not only during cold stress but also before cold treatments (Lee BH *et al*., 2005). This proves that ICE1 is very sensitive to temperature falling and can operate its function very soon.

ICE1 was indicated as down-regulated by HOS1 in many pathways in Group Two: This result may reveal that cold stress responses in Barley are attenuated by an ubiquitination/proteasome pathway in which HOS1 mediates the degradation of the ICE1 protein (Kenji Miura *et al*., 2007).

The variant RING finger protein high expression of HOS1 (High Expression of Osmotically Responsive genes 1) was identified genetically as a negative regulator of

cold responses. And the ubiquitination of ICEL1 requires an E3 ligase which has been identified as HOS1 (Chun-Hai Dong *et al*., 2006). HOS1 can interact with ICE1 physically both in internal and external to modulate the ubiquitination of ICE1.

When treated with cold, HOS1 would most likely to act as over express and it would seems that the cold induces the degradation of ICE1 (Byeong-ha Lee *et al*., 2005).

HOS1 acts to be the key of the degradation of ICE1 when plants are treated with cold temperature. With the degradation of ICE1, the expression of corresponding CBFs and their downstream genes would also be repressed. As a result, the sensitivity to cold and freezing stress of plants would not be increased and eventually cause cold damage (Jianhua Zhu *et al*., 2007).

In all pathways that ICE1 involved, ICE1 appeared as significant down expression according to the Filter on Volcano Plot Analysis. This comparison was made between [root,4℃] and [leaf, 20℃](with root as 4℃), suggesting that the expression of ICE1 would be repressed by HOS1, as a result to attenuate the ability to cope with cold stress of Barley plants.

4.2.4 LTI30 and Cold Acclimation Response

LTI30 is known as LOW TEMPERATURE-INDUCED 30.
LTI30 is generally a symbol gene that would be induced by low temperature.

LTI30 was only found in the pathways of group three [leaf,20℃](with root as 4℃) Vs [leaf,20℃] and involved in the following pathways: Expand Interactions Pathway, Network Targets and Regulators Pathway and Transcription Regulators Pathway. LTl30 was significant up regulated by abscisic acid, a transcription factor and COR47 in the Expand Interactions Pathway.

LTI30 can't be detected in plants that without stress. However, in plants that have been treated with cold, LTI30 is highly expressed and the tolerance to cold of plants is greatly enhanced (Susanna Chung and Roger W. Parish, 2008).

The expression pattern of the five dhn/lea/rab-related genes (COR47, DHNX, LTI30, LTI40 and RAB18) indentified so far was characterized in plants exposed to low temperature (Welin BV *et al*., 1994). LTI30 is located at membrane, and is a member belongs to the dehydrin protein family. LTI30 contains highly conserved stretches of 7-17 residues that are repetitively scattered in their sequences, the K-, S-, Y- and lysine rich segments. LTI29 and LTI30 double overexpressors confer freeze tolerance (Welin BV *et al*., 1994).

In the Network Targets and Regulators Pathway, LTI30 was up regulated by ABA, suggesting that ABA is of central important in the expression of LTI30. And meanwhile, LTI30 mRNA was found to be highly expressed by water deprivation and abscisic acid (Welin *et al*., 1994).

In the Expand Interactions Pathway, LTI30 was indicated as up-regulated by the regulator COR47 (COLD REGULATED 47). COR47, just like LTI30, belongs to the DHN (dehydrin) genes family (Welin *et al*., 1994). COR47 accumulates primarily in response to cold temperature (Tuula Puhakainen *et al*., 2004).

Both COR47 and LTI30 were found significant up expression in the cold acclimation Pathway. This may suggests that these two genes are more sensitive to cold stress response than any other genes in DHN genes family. Their multi-function may be realized by the corresponding encoding proteins on the membrane. This is a hypothesis that could be testified in future work.

4.2.5 RAB18 and the correlation with COR47 and ITL30

RAB18 is known as RESPONSIVE TO ABA 18.

RAB18 was found only in two GO groups: Response to Cold and Cold Acclimation. RAB18 was the gene that expressed most frequently in most pathways of all these groups and involved in the following pathways: Expand Interactions Pathway, Network Targets and Regulators Pathway, Transcription Regulators Pathway.

According to these pathways, RAB18 was the main targets of different kind regulators. Moreover, RAB18 was mainly up regulated by abscisic acid both on expression and transcription level.

Due the up-regulation of RAB18, the Barley plants that treated with low temperature (4℃) may be partly protected by their protective effect on membranes (Manabu *et al*., 1997).

RAB18 was identified as a cold responsive symbol gene that can enhance the cold tolerance of plants (Ji-Yeon *et al*., 2003). On the contrary, however, different from those genes that are mainly induced by low temperature, the expression of RAB18 is highly induced both when the plants are treated with ABA and facing water stress. In this case, RAB18 is only slightly response to cold (Welin BV *et al*., 1994).

RAB18 is a gene that would be induced intensively when plants are treated with low temperature (4 degrees centigrade) (Manabu Ishitani *et al*., 1998). The accumulation of RAB18 was detected very slightly in cold-treated plants when the treated time was very short (Elena-Maria *et al*., 2003). And as the lengthening of time, the accumulation of RAB18 became visible and the cold tolerance of plants was enhanced (Einar Mantyla *et al*., 1995). Though RAB18 was found in many pathways, especially frequent in group one [root, 4℃] Vs [root, 20℃], it didn't act as regulator and always under the control of ABA. This can bring forward the opinion that RAB18

is not very sensitive to temperature falling while together with ABA, it does have correlation with cold tolerance in plants.

RAB18 has been proven to regulate the expression of LTI45 and LTI30 which are induced by low temperature. COR47, RAB18, LTI30, these three genes are all belong to the DNH (dehydrin) protein family, and all considered to be cold responsive (Micheal F. Thomashow, 1999).

Pathways have shown that RAB18 and LTI30 were both expressed only in the pathways of group three [leaf,20℃](with root as 4℃) Vs [leaf,20℃]. And both of them were up regulated by abscisic acid. In fact, RAB18 is more sensitive to abscisic acid while LTI30 is more sensitive to low temperature. As COR47 and RAB18 double overexpressor plants are hold high tolerance to cold stress (Kazuo Shinozaki *et al*., 2006), though COR47 didn't show direct connection with RAB17, the LTI30 was up regulated by COR47 in the same pathways that RAB17 involved in group three [leaf, 20℃](with root as 4℃) Vs [leaf, 20℃]. Moreover, COR47 has been proved to directly increase the cold and freezing tolerance (Michael F. Thomashow, 1999). This point may reveal that Barley plants were low temperature tolerant to some extent.

COR47 and RAB18 were both expressed only in group two [root, 4℃] Vs [leaf, 20℃](with root as 4℃) and group three [leaf, 20℃](with root as 4℃) Vs [leaf, 20℃] but not in group one [root, 4℃] Vs [root, 20℃]. RAB18 was significant down expression in group two while significant up expression in group three. This may suggest that with the temperature falling down, RAB18 is easier to accumulate in leaves than roots and it still need low temperature to induce. The enhancement of cold tolerance in leaves is likely to be stronger than roots. This may due to that RAB18 is considered to realize its protective effort on membranes, and RAB18 is more likely to locate at mesophyll cell than meristem in roots. The reason for this selective location is still unknown and may worth future work.

4.2.6 SEX1 and Starch Catabolic Process

SEX1 is known as STARCH EXCESS 1. SEX1 was only found in group one [root, 4℃] Vs [root, 20℃] within the GO groups: Response to Cold and Cold Acclimation.

SEX1 was found as significant up expression only in Expand Interactions Pathway which illustrates that SEX1 was not acted as transcription regulators or network targets. As can be seen from the pathway, SEX1 has interactions with many molecules. The expression of SEX1 could be depressed by mycose, and SEX1 was an important reactant in the production of starch and lignins. Meanwhile, SEX1 was also an important regulator in the starch catabolic process which is considered as the most important reserve polysaccharide in plants (Michael F. Thomashow, 2001).

SEX1 was not found to be significant in group two [root, 4℃] Vs [leaf, 20℃](with root as 4℃) and group three [leaf, 20℃](with root as 4℃) Vs [leaf, 20℃]. This may suggest that the meristem cells in roots were more sensitive to cold stress response than mesophyll cells, and this cold stress response would result in the breakdown of starch.

Researcher has proposed that the molecular characterization of the Arabidopsis SEX1 mutant was shown as insufficient in the export of glucose (Nakashima Yano *et al*., 2005). This might be caused by the hydrolytic starch breakdown. This perspective along with deep-going research brought about the opinion that SEX1 acts as a comprehensive regulator of starch mobilization by controlling the phosphate content of starch (Tien-Shin Yu *et al*., 2001).

SEX1 encodes a alpha-glucan/water dikinase (EC 2.7.9.4) which is hypothesized to regulate starch degradation in plastids by phosphorylating starch in which way to provide better accessibility by starch-degrading enzymes (Yano R *et al*., 2005).
As SEX1 was not found to be regulated by other genes in all the pathways, it may

evince that SEX1 was most likely to enforce its function as self-contained without involving in the regulation network.

Starch degradation is playing a significant role in cold-induced sugar accumulation (Yano R *et al*., 2005). As research has shown, there is a genetic link between SEX1 locus and plant cold tolerance (Kazuo Nakashima *et al*., 2009). SEX1 is proposed to increase the cold and freezing tolerance of plants through starch degradation (Kyonoshin Maruyama *et al*., 2009).

An inference could be made that when the roots of Barley plants were treated with cold temperature, soluble sugar would accumulate and the accumulation of soluble sugar would enhance the cold stress tolerance in Barley plants. But how long did the soluble sugar need to accumulate can't be inferred from only pathway analysis. However, it's of interest to find out the accumulation time to interpret how SEX1 work during this period and eventually to fulfill the function of SEX1.

As SEX1 was not found as significant expression in group two [root, 4℃] Vs [leaf, 20℃](with root as 4℃) and group three [leaf, 20℃](with root as 4℃) Vs [leaf, 20℃], whether SEX1 has correlation with photosynthesis is unknown.

4.3 Future Work:

- When plants are suffering from cold stress, Propyl Gallate and other kinds of anti oxidant can be removed or limited at very low level. It would be interesting to see if the FAD7 gene overexpressed and silencing can change the cold tolerance of plants in order to prevent plants from cold damage.

- As ICE1 is very sensitive to temperature falling and can response to cold temperature very quickly, it's considered to be a desirable gene to prevent plants from cold damage in advance. The ICE1 mutants would give a better idea of how plants protect themselves from cold damage during a very short period. As the repression of HOS1 is a critical problem for ICE1 to realize its function, methods could be figured out to depress the expression of HOS1.

- RAB18 can enhance the cold and freezing tolerance of plants though it's not very sensitive to temperature falling and need a certain period to induce. From the results of pathways analysis, RAB18 was highly expressed in leaves while was appeared as down expression in roots. This may due to the selective location on membranes of RAB18. In order to prove the expression of RAB18 to give protection to roots, effort would be put on this perspective.

- As many cold-stress response genes and corresponding pathways of Barley have been found, it's accordingly possible to build up the intergration regulatory network with these transciptome data of Barley. To give a detailed and comprehensive view of how Barley Plants react to cold and more photographic insight of how the genes, regulators and molecules interact with each other and eventually to provide useful information in unraveling molecular mechanism associated with abiotic stress response in plants.

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